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13. ABSTRACT (Maximum 200 Words)

The Department of Defense (DOD) post-doctoral training grant was used to support the career development of 3 individuals, Dr. Thomas Boileau, Dr. Peter Carlton, and Dr. Shihua Wang. Dr. Boileau began his initial training at The Ohio State University under the direction of Dr. Tammy Bray (original mentor on this grant) in the Department of Human Nutrition. He initially completed several projects focusing on the relationships of dietary tomato products, lycopene, and energy intake in prostate cancer, including a clinical study proposed in the original application. Midway through the DOD funding period, his primary mentor Dr. Tammy Bray, was recruited to Oregon State University as Dean. Dr. Boileau joined Dr. Steven K. Clinton's laboratory to complete several of his research projects. He was subsequently able to secure an excellent position in industry where he will continue with his research nutrition and prostate health using dog models. At that time, Dr. Peter Carlton was provided support for a few months on the DOD project but soon thereafter, successfully obtained independent funding to support his salary and career development. Dr. Shihua Wang was added to complete the DOD training grant, including the laboratory studies that were proposed as part of the original application. The results of the studies are described in detail in the attached report. In conclusion, through the DOD support, three investigators have achieved valuable training, particularly in exposure to clinical investigation, and enhanced their career opportunities while generating new knowledge that will enhance future prostate cancer research and patient care.

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INTRODUCTION

OVERVIEW. The primary goal of the DOD post-doctoral training award is to facilitate the career development of Ph.D. scientists through research experience, didactic instruction, and mentoring. The DOD project was initially funded at OSU with Dr. Tammy Bray, Chairman of the Dept. of Human Nutrition as the primary faculty mentor and Thomas Boileau, Ph.D. as the trainee. Midway through the funded project, Dr. Bray was recruited to Oregon State University to assume a role as Dean and the DOD grant assigned to Dr. Steven Clinton as primary mentor. The initial trainee, Dr. Boileau was recruited to industry where he continues research on nutrition and prostate health. The DOD support was subsequently used to train two additional individuals: Peter Carlton, Ph.D. and Shihua Wang, Ph.D. Dr. Carlton very quickly received a competitive post-doctoral training award from NIH and Dr. Wang was then recruited to complete the project.

The training program for the post-doctoral fellows in our program included lectures, weekly laboratory group meetings, seminars, one-on-one teaching in the areas of nutrition, biostatistics, and carcinogenesis/chemoprevention. Each trainee was exposed to research activity ranging from molecular and cell biological techniques, rodent models of carcinogenesis, clinical trial design and execution, and epidemiologic studies. Each trainee participated in national meetings and has received mentorship in scientific writing and grantsmanship. Although I feel that the fragmentation of the mentorship and trainees during the funding period was less than optimal, the circumstances were unexpected, and the adjustments made were appropriate. The funding has been well invested in the training of three outstanding individuals that will be productive scientists in the years to come.

DEVIATIONS FROM THE ORIGINAL STATEMENT OF WORK: The change in personnel is described above. In addition the design of the clinical study originally proposed in the DOD application was modified in response to reviews by the OSU Institutional Review Board (IRB), The Clinical Scientific Review Committee (CSRC) and the biostatisticians.

The major changes were as follows. The reviewers felt strongly that the study should be a phase II trial design rather than a randomized phase III study. This was based upon concern for patient safety since no study with these interventions had been conducted in men) with active prostate cancer and having all of the typical co-morbidities found in men in this age group (generally 70 or more). The reviewers were also of the opinion that compliance would be very poor with our dietary interventions (which was proven wrong) and demanded to see data showing compliance prior to having us proceed with a phase III randomized study.

Secondly, the reviewers were concerned that the men randomized to the control group in a potential phase III study would change their diet on their own (similar to what has been seen in studies of hypertension and heart disease) to such a degree as to allow no valuable comparisons.

The study that was revised and conducted was thus changed to a two-arm randomized study (soy vs. tomato products) with cross-over to both interventions simultaneously. The study is described in detail below with preliminary data under "project 5". In addition, after transfer of the grant to Dr. Clinton, we incorporated training objectives for the post-doctoral fellows that included exposure to laboratory *preclinical* and *in vitro* studies. These efforts are shown in "projects 1-4", simply to illustrate to the reviewers that the trainees received a comprehensive training in translational research. In addition, the work illustrates the productivity of the individuals during the funding period on projects scientifically related to the original clinical trial in the SOW (see project 5).

BODY

SCIENTIFIC BACKGROUND

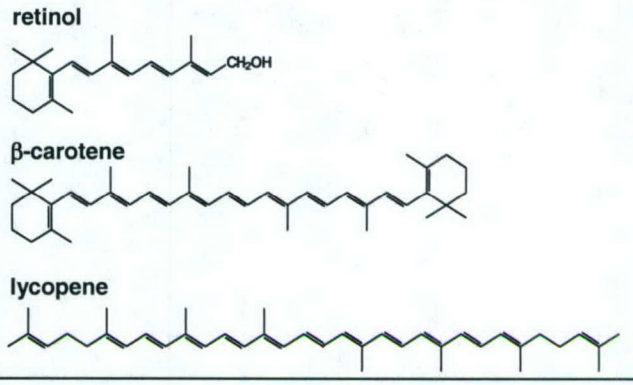
The prostate cancer burden: opportunities for prevention. Prostate cancer is the most common visceral malignancy and the second leading cause of cancer death in American men. In 2004, approximately 230,000 new cases of prostate cancer and 30,000 deaths will be recorded (1). At current rates approximately 9% of American men alive today will be diagnosed with prostate cancer. Although the minority dies of the disease, those cured by early detection and treated with prostatectomy, radiation, brachytherapy, cryotherapy or other modalities, frequently experience lifelong sexual dysfunction and/or urinary incontinence (2-4). Efforts to define preventive strategies, reduce the risk of recurrence after initial therapy, and improve therapy are critically needed. It is our opinion that diet and nutritional interventions may play a role in each of these approaches. For a comprehensive overview of diet, nutrition and prostate cancer, readers are referred to several reviews (5-7).

Tomatoes and prostate cancer: epidemiologic relationships. Detailed reviews have been published regarding prospective cohort, case-control, and clinical studies examining the relationship between prostate cancer risk and the consumption of tomato products (6-8). The landmark study by Giovannucci et al. (9) first reported results from the Health Professionals Follow-up Study (HPFS), a prospective epidemiologic study of over 47,000 men which began in 1986. The only fruit or vegetable food items found to be significantly associated with reduced prostate cancer risk were raw tomatoes (RR = 0.74; 95% CI, 0.58-0.93; *P* for trend, 0.03 for zero servings per wk. vs. 2 to 4 servings per wk.), tomato sauce (RR = 0.66; 95% CI, 0.49-0.90; *P* for trend 0.001 for zero servings per wk. vs. 2 to 4 servings / wk.), pizza (RR = 0.85; 95% CI, 0.45-1.58; *P* for trend, 0.05 for zero servings per wk. vs. 2 to 4 servings / wk.) and strawberries (RR = 0.80; 95% CI, 0.57-1.10; *P* for trend, 0.005 for zero servings per wk. vs. 1 serving / wk.) These results were updated in 2002 (10) and were consistent with the earlier findings. However, a particularly strong association was noted between tomato product consumption and prostate cancer risk in men over 65 years of age (RR for >2 servings tomato sauce/week versus < 1 serving / month, 0.69; 95% CI, 0.56-0.84; *P* for trend, 0.001)(11). Although not all studies agree, the majority of studies suggest a relationship between tomato products and prostate cancer prevention (12). The significance of these associations has been reinforced by our recent study in a carefully controlled rodent model showing an inhibition of prostate carcinogenesis by the incorporation of freeze-dried tomato powder into the diet (13). How much tomato product consumption may be necessary to achieve a lower risk of prostate cancer? The answer is unknown, but based on current data; our best estimate is that 5 servings per week over a lifetime may be associated with a lower risk. Can higher frequency of intake or a shorter duration of intake, such as daily servings of larger quantities of tomato products have an even greater benefit or is there a risk? Again, the answer is unknown.

Tomatoes and prostate cancer: human intervention trials. Unfortunately, there are no intervention trials to define "dose/response" relationships. Two human intervention trials, from the University of Illinois-Chicago (14) and Wayne State in Detroit (15) both employed a fixed dose of 30 mg lycopene per day from a tomato food or a lycopene supplement, respectively, suggested a favorable impact on prostate biomarkers. The team headed by Dr. Phyllis Bowen from the University of Illinois in Chicago demonstrated that the consumption of tomato sauce for 3 weeks before a scheduled prostatectomy (30 mg/day lycopene) led to a lower mean leukocyte 8OhdG concentration in parallel with higher serum and prostate lycopene levels (14). This seminal observation suggested a possible mechanistic link between tomato products, prostate lycopene, and DNA damage. Researchers at the Karmanos Cancer Institute in Detroit randomized 26 men to either a tomato oleoresin supplement (lipid concentrate enriched in lycopene carotenoids, providing 30 mg lycopene/day) or no supplementation for 3 weeks prior to prostatectomy. Men

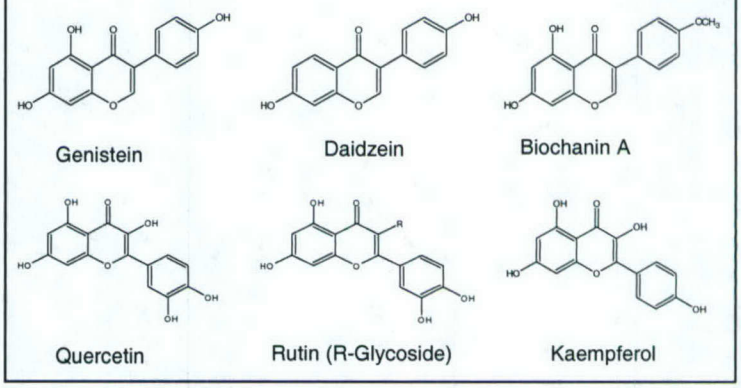
consuming the lycopene supplement were more likely to have no involvement of surgical margins (11 or 73% in the intervention group v. 2 or 18% in the control group, $p = 0.02$) and they were less likely to have diffuse involvement of the prostate by HGPIN (10 or 67% in the intervention group v. 11 or 100% in the control group, $p = 0.05$) (15). A case report suggests an antitumor effect of lycopene in a patient consuming a lycopene supplement (15) (anecdotal data is not convincing). There are currently no human intervention studies that investigate the effects of lycopene at varying doses on biomarkers of prostate carcinogenesis. Thus, we feel that it is essential to conduct studies over a range of tomato product/lycopene intake in order to assess specific dose response relationships for tomato products and biomarkers related to prostate cancer risk.

Figure 1. Lycopene and other carotenoids found in tomato products.



Tomato phytochemicals. Tomatoes and tomato products contain an array of nutrients and non-nutrient phytochemicals that could potentially impact upon prostate carcinogenesis (7,15,16,17). The components most likely to significantly influence prostate carcinogenesis are the carotenoids and polyphenols. Lycopene is the most abundant carotenoid in tomato products accounting for approximately **65%** of the total, while β-carotene, α-carotene, phytofluene, and phytoene among others are also present. Lycopene is a highly unsaturated lipophilic 40- carbon carotenoid ($C_{40}H_{56}$) and is responsible for the red color of tomatoes (**Fig.1**) (7) but lacks the β-ionone ring, and therefore, is devoid of vitamin A activity. Details regarding lycopene's chemistry, absorption, metabolism, tissue distribution, intracellular localization, and molecular effects have been reviewed (7,16,17). A variety of polyphenols, such as quercetin, rutin (a representative glycoside), and kaempherol are found in tomato products and are similar in structure to soy polyphenols (**Fig. 2**). Although compared to carotenoids, much less data is available, published studies have shown tomato polyphenols to exhibit a variety of biological effects, *in vitro* and in rodent models, which could possibly influence susceptibility to carcinogenesis (13,18-20).

Figure 2. Representative tomato and soy polyphenols.



Tomatoes and prostate cancer: hypothesized mechanisms? Most investigators have focused almost exclusively and perhaps excessively, upon lycopene as the most likely candidate for inhibition prostate carcinogenesis (21). Our laboratory was the first to document the presence of lycopene and its isomers in the human prostate and this has now been reported by five other laboratories (14,22-24). The team from the University of Illinois in Chicago demonstrated that the consumption of tomato sauce for 3 wks (30 mg/day lycopene) led to a lower content of 8OhdG concentration in parallel with higher serum and prostate tissue lycopene levels. This seminal observation suggested a possible mechanistic link between tomato products, prostate lycopene, inhibition of oxidative stress and reduced DNA damage (14, 25). Our laboratory was the first to

address the ability of tomatoes (freeze dried tomato powder) or pure lycopene to inhibit prostate carcinogenesis in a laboratory animal model (13). We examined prostate cancer specific survival using the NMU-androgen induced system in rats, a system that mimics many aspects of human prostate carcinogenesis (26). We observed a highly significant benefit for dietary tomato powder at 10% of the diet on survival compared to rats fed the control diet. Although the lycopene fed rats showed a trend towards slightly lower risk of death, this effect was not statistically significant. We conclude that tomatoes may contain components *in addition to lycopene* that may inhibit prostate tumorigenesis in the rodent model. Our laboratory has also initiated studies focusing upon mechanisms whereby tomato polyphenols inhibit prostate cancer cell proliferation, enhance sensitivity to apoptosis, specifically block IGF-I-induced proliferation and survival, and inhibit intracellular signaling cascades involving Akt (27).

In summary, it is premature to conclude that tomato products, lycopene, or other components in tomatoes will inhibit prostate carcinogenesis or serve as an adjunct to therapy (8). However, we feel that the data generated from human epidemiology, intervention trials and rodent models provide strong evidence supporting continued research to test this hypothesis. Our work, and that of others, suggests that studies of food based interventions (tomato products) as well as specific phytochemicals (such as exploring the possibility that lycopene may act as a chemopreventive agent) should continue rapidly. Potential mechanisms whereby these components may act, either individually, or in combination should be addressed.

Lycopene and carotenoids: absorption, tissue distribution, and metabolism. Until the last decade, little research focused upon lycopene absorption, bioavailability, tissue distribution, isomerization, and metabolism. Our knowledge in these areas remains superficial (28-30). However, several key points have emerged. Lycopene is primarily in the all *trans* form in commonly consumed tomato varieties and food products made from them (30). Although lycopene is unstable under certain conditions, it is remarkably stable within the food matrix and during typical processing and cooking procedures (30, 31). Many variables, such as food processing, meal size and composition (such as fat and fiber content), and cooking methods will influence digestion and bioavailability of lycopene (32-34). Our laboratory has determined the lycopene clearance rate is 12-14 days in the majority of healthy men and women when placed on a lycopene free diet (34) although past studies have been extremely inconsistent (32). We have also observed that lycopene absorption is primarily via chylomicrons and reaches circulation after liver processing into lipoproteins (35). Our rat studies suggest that testosterone influences liver lycopene metabolism although this has not been investigated in humans (36,37). Our data suggests that blood concentrations of lycopene can change very quickly in response to a change in intake (for example, daily standard serving sizes) with a new steady state lycopene concentration in the blood achieved after 4-8 wks of feeding. Interestingly, lycopene in blood and tissues is primarily in multiple *cis* isomers (approximately 60% in blood and 85%in tissue), (38-40) which is also observed in animal models (36,37). How *cis* isomerization occurs and how isomerization is related to biological effects remains unknown. Our ongoing studies suggest that *cis* isomers are cleared at different rates compared to all-*trans* (34). Several laboratories, including those of Kachik (41) have begun to elucidate the metabolic degradation products of lycopene but this area is in large part a mystery. We do not know what factors influence degradation processes, where or how they occur, or how this information may suggest biological processes that involved lycopene, or even if degradation products have biological effects. Thus, additional, well designed studies focusing upon tomato products, carotenoid absorption, tissue distribution, and metabolism in parallel with biological outcomes are needed.

Tomato polyphenols: absorption, tissue distribution, and metabolism. There is relatively little published data on tomato polyphenols regarding estimated intake, absorption, metabolism, and biological effects in humans (41-43). The major components are quercetin and

kaempferol which are found naturally as conjugates (**Fig. 2**). Rutin (quercetin 3-rhamnosylglucoside) is the main conjugated form of quercetin in tomato. Quantitative analysis reveals that total free and conjugated polyphenols range from 1.3 to 22.2 μg/g of fresh weight in different tomato varieties (44). In another study, quercetin content varied from 2.2 to 203 of μg/g fresh weight in different tomato varieties (45). The distribution of quercetin in the tomato is uneven. More than 98% of the conjugated quercetin is found in skin, compared to only 1% is in the seeds and flesh (44). It is important to keep in mind that most processed tomato products, including juice, is reconstituted from paste prepared with all tomato components (**Table 1**) (44). Thus a single standard serving of tomato juice (250 ml =1 cup) provides as much as 4 mg of polyphenols.

The glycoside of quercetin is more efficiently absorbed (52%) than the aglycone (24%) (46-47). The absorbed polyphenols are subsequently metabolized and excreted through glucuronidation, sulfation and methylation (48,49). Very few studies have attempted to examine blood or tissue concentrations (46, 50-52). Plasma peak concentrations after single meal ingestion of quercetin-3-glucoside occurs at just over 30 min (53). Half-life elimination time of quercetin from blood is 18-24 h after ingestion of quercetin-3-glucoside(49,52). Most of the absorbed or unabsorbed polyphenols are excreted through the feces, while a small amount (less than 2%) of quercetin and rutin are found in urine (49).

Table1. The estimated amount of polyphenol consumption from tomato products based on serving size.

Tomato product	*Polyphenol content*	*Serving size*	*Polyphenols per serving*
Juice	14-16 μg / mL	1 cup (250 ml)	3500 -4000 μg
Soup	1-2 μg / mL	1 cup (250 ml)	250 to 500 μg
Ketchup	33-41μg / g	1 tablespoon (15 g)	495 - 615 μg
Sauce	9-10 μg / g	½ cup (123 g)	1107 - 1230 μg

Soy and Prostate Cancer Risk. The concept that soy may have anticancer properties is derived from many sources, including epidemiology, clinical biomarker studies, rodent studies, as well as *in vitro* investigations. This work has been the subject of many reviews (53-56). Asians consuming higher amounts of soy, experience relatively low rates of prostate cancer and their emigrants adopting "Western" dietary patterns demonstrate an accelerating risk over time (57-61). Asians consume soy products, such as tofu, approximately 10 times more frequently than Americans (62). Even within nations, some studies suggest that men consuming soy have a lower risk of prostate cancer. Both a prospective cohort study in the Seventh-Day Adventist Health Study and a cross-sectional study have shown that soy milk or soy product consumption is inversely associated with risk (63). For example, soy milk consumed more than once per day reduces the risk by 70 % (RR = 0.3, p= 0.03).

Several investigators, including our own laboratory group, have reported data suggesting an inhibition of prostate carcinogenesis in rodent models. We observed that an isoflavone enriched soy phytochemical concentrate (1 % of the diet) or soy protein (20 % of diet) inhibits the growth of transplanted androgen sensitive human LNCaP prostate cancer in nude mice (64), which was also observed by Aronson et al. (65). Others have documented inhibition of growth of the Dunning R3327 transplantable prostate adenocarcinoma in the rat (66) with 33% soy flour. Feeding soy protein isolate or genistein reduces the incidence of spontaneous rodent prostate cancer in Lobund-Wistar (L-W) rats (66) and the incidence of the poorly differentially prostate adenocarcinoma in the TRAMP model (67). Diets containing soy prolongs the latency of rat prostate cancer induced by MNU (66), and reduces the prostate tumor number induced by 3,2'-dimethyl-4-aminobiphenyl (DMAB) and testosterone propionate (68). Intraperitoneal

administration of biochanin A significantly inhibits the growth of transplanted LNCaP tumor growth in nude mice (69). Overall, the majority of rodent studies described above, although not all support continued efforts to translate these positive findings into human clinical trials.

Soy contains a variety of components that may have "anticancer" activity in laboratory studies. Most of the attention has focused upon genistein and daidzein which are the predominant isoflavones found in soy at concentrations of approximately 1-3 mg/g (70). In addition, protease inhibitors (Bowman-Birk inhibitor), inositol hexaphosphate (phytic acid), lignans, phytosterols and saponins found in soy products may also have *in vivo* bioactivities relevant to the inhibition of carcinogenesis (71-74). Several studies suggest that soy components have "antioxidant" properties based on circumstantial evidence using various surrogate markers of oxidative stress (75-77). It is also likely that soy components may alter the host hormonal environment. Soy isoflavones demonstrate agonist/antagonist effects on several steroid receptors (78). In *vivo* studies show that dietary genistein dose-dependently reduced the content of androgen receptor (AR) mRNA in the dorsolateral prostate of Male Sprague-Dawley rats (79) and we have observed a reduction in circulating IGF-I in rodents fed soy (80). In addition, the interactions between various cell types and matrix in the tumor microenvironment may also be a target of soy components. Soy polyphenols (genistein or daidzein) or soy phytochemical concentrate has been shown to inhibit *in vitro* proliferation of vascular endothelial cells at low concentrations (80). We have observed that mice fed soy components demonstrate reduced microvessel density in transplanted prostate cancer xenographs (80).

We observed that soy polyphenols (genistein and daidzein) and soy extract dramatically inhibit the *in vitro* growth of both androgen sensitive and insensitive LNCaP, DU145 and PC3 cell lines and confirmed in LNCaP cells (80). Our recent study shows that genistein, biochanin A and daidzein exhibit slightly different potency for the inhibition of proliferation (cell cycle arrest) stimulated by IGF-I in rat AT6.3 prostate cancer cells (27) as has been observed in similar studies by others (69, 81,82) Cell cycle inhibition occurs in parallel with a lowering of the apoptotic threshold for prostate cancer cells (69, 81,83,), which has been observed by our laboratory and others (80, 68, 83, 84). Our *in vivo* biomarker evaluations also show a dramatic effect on apoptosis (increased TUNEL staining) of tumor cells in mice fed soy components, consistent with the *in vitro* observations (80).The cell cycle effects are proposed to occur through several critical regulators, such as: inhibiting cdc2 kinase activity (85), decreasing cyclin B1 levels (86) or increasing expression of CDK inhibitor $P21^{cip1}$ and $P27^{KIP1}$ (69, 81, 86-89). We are convinced that the anti-proliferative and pro-apoptotic effects of isoflavones in prostate cells are due in part, to inhibition of growth factor associated tyrosine-kinase signal transduction (89-93). We recently reported that genistein, biochanin A and daidzein inhibit both constitutively active ERK1/2 phosphorylation and IGF-I induced Akt and IRS-1 tyrosine phosphorylation (27).

Soy phytochemical pharmacokinetics and metabolism. Genistein and daidzein exist primarily as glycosides in soy products. Absorption of isoflavone glycosides is poor and requires deconjugation to release the bioactive aglycone (94). The mechanism of deconjugation remains controversial. It is known that gut bacterial β-glucosidases (primarily in the distal gut) can cleave the glycones (95). However, our preliminary data at OSU (Dr. Mark Failla, Chair, Department of Human Nutrition) suggests that significant cleavage may occur in the upper gut during digestion through uncharacterized mechanisms. Food processing, such as fermentation, may increase formation of the aglycone (96). Pharmacokinetic studies suggest that the maximum blood concentration of daidzein and genistein are achieved within 6-8 h after the consumption of soy polyphenols (97,98) with a plasma half-life of plasma genistein and daidzein range from 3-4 hr (99,100). The polyphenol concentrations significantly increase in rat brain, liver, mammary, ovary, prostate, testis, thyroid and uterus after soy production consumption (99). Almost 100% of the polyphenols found in tissues are present as the physiologically active aglycone form (99).

Interestingly, the concentrations of genistein and daidzein in plasma and prostatic fluid are much higher in Asian men compared with western country populations (101-104). Japanese men have serum concentrations of isoflavones which were approximately 15 times higher than British men (101). The absorbed aglycones are excreted by reconjugation, mainly by glucuronic acid with small amount by sulfate (105), with some conjugates excreted through the liver and bile with a significant proportion undergoing renal excretion into the urine (106). The average 24 h urinary recovery of ingested genistein and daidzein is approximately 25% of consumption (106) with the remainder of ingested polyphenols recovered in the feces (95, 98).

Description of the original SOW and deviations from the original SOW. Slight changes in the SOW have been incorporated and discussed under each task. The original SOW included 4 tasks defined below as Tasks 1 through 4.

Task 1. ***Develop pertinent aid documents for the study***. This includes developing a lycopene content of tomato product spreadsheet, developing a food frequency questionnaire for tomato products, and design of patient spreadsheets to aid in data collection.

Comment. Each of these was accomplished and accurate data regarding tomato and soy intake obtained (see tables 2-3 below under project #5).

Task 2. ***Gain local IRB approval***. Obtain necessary documents and prepare IRB approval.

Comment. The study documents were prepared, submitted to the OSU IRB, revised, resubmitted and approved by the IRB with annual evaluations completed and approved. The study design was modified based upon reviewers concerns and comments. We changed the study from a randomized phase III trial of soy vs. tomato products focusing upon disease progression as the primary outcome (based upon PSA as a surrogate marker) to a randomized phase II study focusing upon safety and efficacy, in addition to evaluation of various biomarkers such as PSA, hormones, carotenoid patterns, lycopene isomers, antioxidant stress biomarkers, and soy isoflavone metabolism. The revised design is shown in the figure below under project #5.

Task 3. *Literature review and assay development*. Complete literature searches, document the state of the art assays for measuring antioxidant stress response and biomarkers.

Comment. The assays to be completed on the dietary records, blood, and urine are established in our laboratories at OSU (carotenoid profiles, lycopene isomers, blood antioxidant capacity, urinary and blood isoflavones and metabolites, serum hormones). The supporting literature shown below under REFERENCES provides evidence that our program has established the assays that will be used on the samples from the clinical trial. For many reasons it is best to evaluate the banked samples in batches, rather than run one at a time as patients complete the intervention.

Task 4. ***Data analysis, present research and write manuscript***. Data analysis, present research and write manuscript.

Comment. The recruitment, completion of the 8 wk intervention study, and collection of biological samples has now been achieved in 38 men. We have the last 2 men enrolled in early December 2004 and all samples should be collected in a few weeks. All remaining assays will be completed in early 2005 and a manuscript prepared by late spring 2005. An interim analysis of some key data is shown under Project #5 below.

KEY RESEARCH ACCOMPLISHMENTS. The first four projects were not part of the original SOW but illustrate some of the accomplishments of the 3 fellows supported by the DOD training grant. Project 5 describes the clinical trial that was the basis of the SOW in the original DOD training grant.

Project 1. Altered nuclear morphometry, loss of androgen receptor, and increased phospho-AKT during *N*-methyl-*N*-nitrosourea-induced prostate carcinogenesis.

Androgens play an essential role prostate carcinogenesis although the loss of androgen sensitivity is characteristic of advanced disease. Mutations in the androgen receptor (AR), activation of growth factor signal transduction pathways by oncogenes, and the development of alternative signaling pathways bypassing the AR are among the mechanism underlying with the development of androgen independent prostate cancer. We examined the interrelationships between AR and p-AKT expression by immunohistochemical (IHC) staining during *N*-methyl-*N*-nitrosourea (NMU)-androgen-induced prostate carcinogenesis in rats. In addition, we employed histone nuclear staining and image analysis to assess parallel changes in nuclear morphology. AR staining was consistently detected in all normal prostate epithelial compartments although the ventral lobe (46 ± 28%, mean ± SD) exhibited a significantly lower ($P<0.01$) proportion of AR positive nuclei compared to the dorsolateral lobe (80 ± 27 %), seminal vesicle (89 ± 9%), and coagulation gland (anterior lobe) (89 ± 17%). Normal prostate epithelia, hyperplasic and atypical hyperplasic lesions all showed detectable AR expression (100% of samples). In contrast, ninety percent of prostates with well differentiated adenocarcinoma, 50% with moderately differentiated and 28% with poorly differentiated adenocarcinoma showed positive AR staining. Among AR positive lesions, the percentage of AR positive nuclei also decreased ($P<0.01$) as prostate carcinogenesis progressed: hyperplasia (92 ± 6 %), atypical hyperplasia (92 ± 9%), well differentiated adenocarcinoma (57 ± 30%), moderately differentiated adenocarcinoma (19 ± 18%), and poorly differentiated adenocarcinoma (10 ± 18%). p-AKT staining increased significantly during prostate carcinogenesis. Sparse staining for p-AKT was observed in normal tissues (0.2 ± 0.3 % of epithelial area) and hyperplastic lesions (0.1 ± 0.1%), while expression increased significantly ($P<0.001$) in atypical hyperplasia (7.6 ± 9.7%), well-differentiated adenocarcinoma (16.7 ± 14.7%), moderately-differentiated adenocarcinoma (19.6 ± 9.7%) and poorly-differentiated adenocarcinoma (17.4 ± 12.8%). Nuclear morphometry revealed significantly greater nuclear irregularity, increased nuclear size, and lower DNA compactness as prostate cancers became more poorly differentiated. Our observations regarding the loss of AR and the increase in p-AKT expression in parallel with changes in nuclear morphometry mimic findings suggested in human studies. AR and p-AKT staining as well as nuclear morphometry may be useful biomarkers in studies assessing the ability of dietary interventions and chemopreventive agents to alter NMU-induced prostate carcinogenesis.

Project 2. Prostate carcinogenesis in N-methyl-N-nitrosourea (NMU)-testosterone-treated rats fed tomato powder, lycopene, or energy restricted diets

Background. Consumption of tomato products, lycopene, and energy restriction have been hypothesized to reduce the risk of human prostate cancer. We investigated the effects of these dietary variables in a rat model of prostate carcinogenesis. *Methods.* Male rats in which prostate cancer had been induced by N-methyl-N-nitrosourea and testosterone treatment were fed diets containing whole tomato powder (13 mg lycopene /kg diet), lycopene beadlets (161 mg lycopene/kg diet) or control beadlets. The three dietary groups were randomly subdivided to either 20% diet restriction or ad libitum feeding. Differences between Kaplan-Meier survival curves for diet composition or restriction were tested with the log-rank, WIlcoxan, Tarone-Ware, and Peto-Peto tests. Cox proportional hazards models were developed to examine the combined effect of

diet composition and restriction on survival. Statistical tests were two-sided. *Results.* Risk of death with prostate cancer was lower for rats fed the tomato powder diet than for control rats (R= 0.55, 95% confidence interval [CI] = 0.35 to 0.86, P = 0.009). In contrast, prostate cancer specific mortality of the control and lycopene fed groups was similar (P=0.63). The proportions of rats dying with prostate cancer in the control, lycopene, and tomato powder groups were 80% (95% CI = 68% to 89%), 72% (95% CI = 60% to 83%), and 62% (95% CI = 48% to 75%), respectively. Dietary restriction modestly delayed prostate cancer specific survival (R = 0.68, 95% CI = 0.49 to 0.96, P = 0.29). The proportion of rats developing prostate cancer was 79% (95% CI = 69% to 86%) for ad libitum-fed rats and 65% (95% CI = 54% to 74%) for rats fed restricted diets. No interactions were observed between diet composition and dietary restriction. *Conclusion.* Consumption of tomato powder but not lycopene statistically significantly inhibited prostate carcinogenesis, suggesting that tomato products contain compounds in addition to lycopene that favorably modulate outcome. Diet restriction also reduced the risk of prostate cancer. Tomato phytochemicals and diet restriction may act by independent mechanisms.

Project 3. Mechanisms regarding IGF-I stimulation of prostate cancer progression through exploration of gene expression profiles.

Epidemiologic studies, rodent experiments, and *in vitro* investigations suggest that IGF-I is a critical growth factor stimulating prostate carcinogenesis and may mediate dietary effects on prostate cancer risk. The purpose of this study was to identify patterns of gene expression whereby IGF-I may enhance prostate cancer cell biology using microarray technology. We isolated mRNA from AT6.3 rat prostate adenocarcinoma cell lines (n=6 for each condition), treated for 24 h in serum free media (SFM), SFM with 10% FBS (serum), or SFM with 50 ng/ml IGF-I (IGF-I). Gene expression profiles were examined by Affymetrix GeneChip U34A and analyzed by dChip and SAM software. IGF-I alone can mimic many of the pro-carcinogenic effects of complete serum stimulation on patterns of genes related to cell cycle control (PCNA, cyclin G1 and P27), proliferation (RCL, transferrin receptor, ODC and PPAR γ), survival (Bax, DAD-1), angiogenesis (VEGF, pleiotrophin), as well as invasion and metastasis (C-FABP, MMP-2, TIMP-2). One critical finding was that IGF-I activates a pattern of genes involving glucose uptake and anerobic glycolysis consistent with the Warberg effect. IGF-I activates critical regulatory genes such as GLUT1, hexokinase II, phosphofructokinase, lactate dehydrogenase, and glucose-6-phosphate dehydrogenase while enzymes involved in the Kreps cycle were not changed. We subsequently showed that 2-deoxyglucose, a metabolic antagonist of glucose, blocked the ability of IGF-I to stimulate cell proliferation and survival suggesting that activation of the Warberg effect may be a critical and essential component of IGF-I-regulated patterns of gene expression that enhance prostate carcinogenesis. These studies identify patterns of gene expression that can be examined and characterized as biomarkers in future rodent and human studies evaluating hypotheses regarding diet and endocrine interactions in prostate carcinogenesis.

Project 4. Genistein inhibits VEGF- mediated autocrine and paracrine pro-angiogenic growth factor networks between prostate cancer cells and vascular endothelial cells

We have reported that dietary soy products inhibit murine prostate tumor progression in association with a reduction in tumor microvessel density. VEGF stimulates angiogenesis by interacting with fms-like tyrosine kinase-1 (FLT-1) and kinase insert domain-containing kinase (KDR) receptors on endothelial cells. Moreover, tumors are known to be hypoxic and hypoxia can further stimulate tumor angiogenesis through the regulation of VEGF and the transcriptional factor, hypoxia-inducible factor-1α (HIF-1α), as well as VEGF receptors in tumor cells and endothelial cells. The goal of the present investigation is to investigate the hypothesis that genistein, a soy isoflavone inhibits the VEGF autocrine and paracrine network in human prostate cancer cells and vascular endothelial cells. By employing MTS and an *in vitro* tube formation assay, we find that genistein inhibits growth and tube formation of human umbilical vein endothelial cells (HUVECs) stimulated by exogenous VEGF or by hypoxia (5% CO_2, 1% O_2, 94% N_2) exposed PC-3 cell media.

Genistein causes a dose-dependent inhibition of VEGF expression in both human prostate cancer PC-3 cells and HUVECs with or without exposure to hypoxia based on RT-PCR and western blotting assays. Furthermore, genistein reduces the expression of HIF-1α in response to hypoxia on PC-3 cells and the expression of Flt-1, but not KDR on the HUVECs. Overall, these observations support the hypothesis that soy isoflavone genistein may inhibit prostate tumor angiogenesis through VEGF-mediated autocrine and paracrine networks.

Project 5. OSU CLINICAL TRIAL (IRB# 2001C0007): A study of adherence to dietary intervention with lycopene-rich foods or soy protein in men with recurring asymptomatic prostate cancer.

Each of the fellows supported by the training grant contributed to, and are continuing to work on the phase I-II randomized clinical trail to assess safety and efficacy of combined soy and tomato products for men with prostate cancer.

Study Objectives: A clinical study was designed to determine if men with asymptomatic prostate cancer and a rising PSA will consume a soy-based dietary supplement (40 g protein / day to provide approximately 80 mg isoflavones / day for 4 weeks), tomato products (to provide a minimum of 25 mg lycopene / day for 4 weeks), or a combination of the soy supplement diet with a tomato product enriched diet (see the figure below). The IRB and team of investigators felt that the original design of a phase III randomized trial of soy vs. tomato vs. usual diet was inappropriate for several reasons: (1) underpowered with n=6 to achieve efficacy outcomes, (2) neither soy powder or high intake of tomato products had previously be tested in the older prostate cancer patient population for safety and compliance. Thus we changed the design to a phase II trial focusing upon compliance, safety, and impact upon biomarkers.

The primary outcome is compliance and safety (toxicity) to either a lycopene-rich tomato product diet, soy protein supplemented diet, and a combination of both diets (soy + tomato products). Compliance will be measured by food diaries and interviews and by biochemical markers of lycopene or soy intake (e.g., blood lycopene concentrations and urinary excretion of soy isoflavones) along with common toxicity criteria.

In addition, secondary goals of this study are to determine the effects of dietary interventions on (a) blood carotenoid profiles and lycopene isomers, (b) blood antioxidant capacity, (c) blood testosterone and insulin-like growth factor I (IGF-I) concentrations, (d) serum prostate specific antigen (PSA), and (e) lipid profiles.

Eligibility requirements: The study population selected takes advantage of the large clinical population at OSU with prostate cancer that are showing "biochemical failure" meaning an asymptomatic rising PSA after either localized therapy (prostatectomy, external beam irradiation, brachytherapy) or hormonal therapy. Although this population is destined to show clinical progression, the rise in PSA may precede symptomatic disease by months or even many years. Thus, many men in this population are ideal for testing several of the dietary and nutritional interventions that are hypothesized to reduce the rate of progression or complement other treatments. Issues of compliance, safety, and perhaps efficacy (based on surrogate markers such as PSA or hormones) can be evaluated.

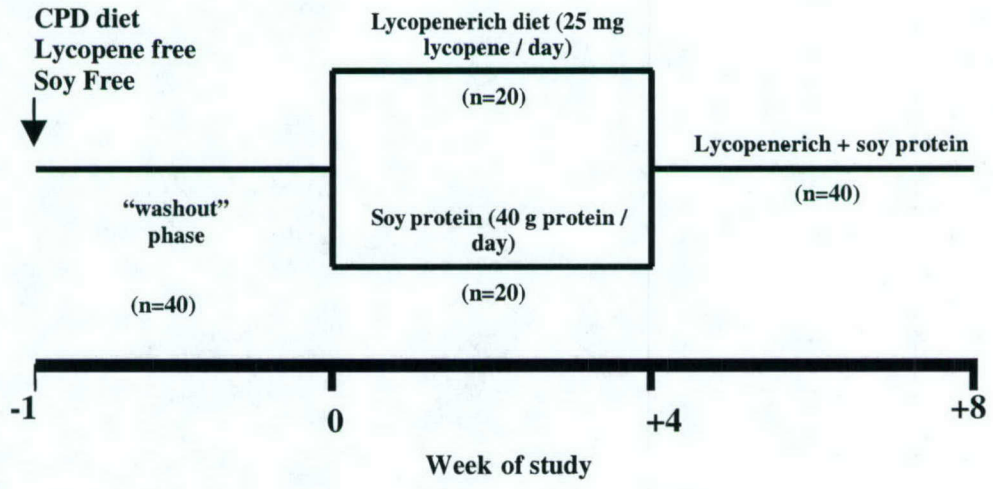
To be eligible for this study, participants must:

- Have histologically confirmed prostate cancer diagnosis.
- Have completed primary therapy (radical prostatectomy, external beam radiation, brachytherapy) or chosen "watchful-waiting" and are currently demonstrating biochemical failure (rising PSA).

- Are not receiving ongoing or concurrent therapy of "alternative" medications such as PC-SPES that may influence hormone profiles.
- Be asymptomatic and based on clinical and laboratory assessment are expected to remain so for at least 3 mo.
- Have demonstrated a rise in PSA on two serial samples after a nadir following primary therapy. If the patient has chosen "watchful waiting" two consecutive increases in PSA will allow enrollment. There is no minimal concentration of PSA required or maximum PSA allowed. A second group showing asymptomatic rising PSA after adequate hormonal therapy (GNRH antagonists and oral antiandrogens).
- Not be receiving ongoing chemotherapy, radiotherapy, or biological therapy for an internal malignancy including prostate cancer. Patients must not undergo changes in hormonal therapy during the study.
- Have kidney and liver enzymes within normal limits.
- Have no history of malabsorptive disorders or other metabolic disorders requiring special diet recommendations. Men with type I or type II diabetes mellitus will be permitted to participate.

Patients are recruited from the urology, radiation therapy, and medical oncology clinics at the Arthur G. James Cancer Hospital and Richard J. Solove Research Institute. It is not anticipated that other sources of participants outside of OSU will be needed to achieve the trial goal of 40 men.

Design: The study requires 40 men that will have one week of "washout" without consumption of tomato products or soy products prior to randomization to one of two dietary interventions with 20 men per group, (a) tomato products providing a target of at least 25 mg lycopene per day, or (b) soy protein at 40 g per day (**Fig. 1**). These will be consumed for 4 weeks. After 4 weeks all men receive a combination of both soy and a tomato-rich diet for an additional 4 weeks. Blood and urine samples are obtained at randomization, after 4 weeks of intervention on single agents, and after 4 weeks on combined product intervention.



Washout Phase. All 40 men will be asked to consume diet devoid of tomato products or soy for 7 days. This is necessary to establish a new baseline for blood lycopene and urinary soy isoflavones. Men will avoid lycopene-rich fruits and vegetables (tomatoes and tomato products, watermelons, red grapefruit) and soy products (tofu, soymilk and shakes, and soy nutritional supplements). In earlier studies we have shown that lycopene concentrations will decrease by approximately 50% in 7 to 14

days of "washout". Men are then randomized after 1 wk of the "washout" phase to either 4 weeks of dietary intervention with soy products or tomato products. Men are then randomized after the one week washout to receive either a lycopene-rich tomato product diet (targeting 25 mg lycopene / day)(n=20) or the soy protein (40 g / day)(n=20) supplement for 4 weeks. In the second 4 weeks of the study, all patients receive a combination of the lycopene-rich tomato diet and the soy supplement diet. Blood and urine samples are collected at the end of washout/randomization, after 4 weeks of intervention, and after 8 weeks of intervention.

Lycopene enriched diet. The lycopene-rich foods are selected from an instructional list providing a "lycopene score" and should be consumed in frequency and amount to provide a minimum of 25 mg lycopene / day. We have chosen the minimum of 25 mg lycopene / day based on epidemiologic studies in which a 35% reduction in risk in men who consumed >10 servings of tomato products / week versus those who consumed <1.5. Based on lycopene analysis of tomato products (19), 10 servings tomato products / week provides approximately 150 mg lycopene / week or 21 mg lycopene / day. Our study will target 25 mg lycopene / day from a variety of food sources (tomato sauce, tomato soup, tomato-based juice, whole tomatoes, pizza, etc.). Our data suggests that 25 mg lycopene / day is approximately 1-2 servings of lycopene-rich food / day and should be very achievable by most participants. A trained dietitian instructs participants about ways in which lycopene-rich foods can be creatively incorporated into their daily meals.

Soy enriched diet. The soy protein supplement from Protein Technologies International (St. Louis, Mo.) is a standardized product that can be mixed with milk, juice, water or other drinks. Products from PTI have been used in the majority of published studies of dietary soy and health undertaken at American academic centers. The rationale for choosing 40 g soy protein / day for our soy treatment is based on a recent study by Teixeira et al (28) in which it was shown that 40 g isolated soy protein (ISP) / day for 3 weeks is the minimum amount of soy protein needed to achieve blood and urine isoflavone concentrations in men that are similar to those found in Asian populations (29, 30). We provide 40 g soy protein isolate in the form of 2 daily packets of 20 soy protein powder packets which can be mixed in milk, water or juice and easily consumed.

Combination of soy and tomato enriched diets. After the randomized phase of the study lasting 4 wks, we will provide a combination of both soy and tomato enriched diets to all participants for an additional 4 wks.

Blood and urine collections. Blood samples (approximately 30 cc) and urine (24-hour sample) are collected at three time points, including (a) the end of "washout", (b) after 4 wks of intervention, and (c) after 8 wks of intervention. Samples are processed using standardized sterile techniques in Dr. Clinton's laboratory for analysis of biomarkers.

Preliminary Results. Some of the interim data can be shown and is summarized in the tables below. Table 1. shows that the recruitment resulted in a homogeneous population of men that is typical for men participating in prostate cancer clinical trials. There were no statistically significant differences in clinical parameters of men randomized to the two groups. We employed an FFQ to define the usual dietary patterns for the men on the study, some of the data is shown in table 2. No major differences in nutrient intake between the two dietary groups was observed. Table 3 shows the data for compliance using our assessment tools created for the study. The men on this study did a superb job in consuming the soy product and with our education and consultation found many ways to incorporate the supplement into their diets with outstanding compliance. We targeted a minimum of 25 mg/day of lycopene intake from tomato products and the men exceeded our expectations with intake significantly higher in most men. We saw not significant decline in compliance where the two arms were combined (soy plus tomato). Using NCI toxicology criteria we

observed no significant side effects except for grade I constipation in 10% of men consuming the soy product. Preliminary analysis of IGF-I has been completed and no change in the serum concentration of the hormone is suggested. Most interestingly, the preliminary data on PSA is intriguing. As you may expect, absolute PSA values of men entering the study ranged from approximately 1 to over 1000, making simple ANOVA/T-testing inappropriate. Thus we will be evaluating PSA velocity prior to enrollment and during the study. Of interest is the initial categorization of this data shown in the final table. Although we would not expect that soy or tomato products would act like "chemotherapy" where a 50% decline in PSA is considered a response, the change in slope of the PSA rise is of interest and we await the final two participants and a complete analysis of the data.

Ongoing Work. The recruitment of subjects and their study interventions have been completed. Currently, carotenoid analysis is being accomplished and should be completed by April 2005 (40 men x 3 samples = 120 total). Additional analysis will include: lycopene isomers, urinary soy isoflavones, and blood antioxidant capacity.

Project #5, Table 1: Characteristics of the first 38 men (subjects 39 & 40 in progress) enrolled in the study showing that the two groups are comparable.

	Tomato Group	**Soy Group**
	mean ± SD	mean ± SD
Anthropometrics		
Age (years)	66 ± 7	72 ± 7
Height (inches)	70 ± 3	70 ± 2
Weight (pounds)	210 ± 32	189 ± 32
Body Mass Index (kg/m^2)	30 ± 4	28 ± 5
Vitals		
Resting Pulse (beats / min)	73 ± 11	77 ± 13
Systolic Blood Pressure (mm Hg)	138 ± 13	137 ± 16
Diastolic Blood Pressure (mm Hg)	80 ± 8	76 ± 10
Clinical State		
No Hormone Therapy	n = 11	n = 8
Hormone Insensitive	n = 9	n = 10
Laboratory Values		
Chemistry		
Glucose (mg/dL)	118 ± 28	113 ± 37
BUN (mg/dL)	21 ± 8	17 ± 7
Cr (mg/dL)	1.1 ± 0.18	1.0 ± 0.2
ALT (U/L)	22 ± 6	21 ± 6
AST (U/L)	24 ± 5	24 ± 5
Bili (mg/dL)	0.7 ± 0.4	0.7 ± 0.3
Hematology		
WBC (k/uL)	6 ± 2	7 ± 3
Hct (%)	42 ± 3	41 ± 4
Hgb (g/dL)	14 ± 1	14 ± 1
Platlets (K/uL)	239 ± 49	278 ± 80
Lipid Profile		
T. Cholesterol (mg/dL)	190 ± 39	191 ± 32
HDL (mg/dL)	45 ± 21	53 ± 34
LDL (mg/dL)	114 ± 33	108 ± 32
Triglycerides (mg/dL)	164 ± 94	153 ± 61

Project #5, Table 2. Estimated dietary composition of the first 38 men randomized to one of the two dietary groups demonstrating similarity.

	Tomato Group	**Soy Group**
	mean ± SD	mean ± SD
Daily Kcal	1973.2 ± 576.7	2019.8 ± 545.9
Protein (g/d)	89.1 ± 23.4	86.3 ± 24.0
CHO (g/d)	243.1 ± 92.5	279.2 ± 78.1
fiber (g/d)	22.2 ± 5.5	25.1 ± 9.0
fat (g/d)	69.5 ± 30.0	63.9 ± 21.1
sat fat (g/d)	22.9 ± 11.5	21.1 ± 9.9
chol (mg/d)	318.7 ± 174.1	237.9 ± 98.3
Vit A (RE/d)	1304.8 ± 648.7	1687.6 ± 1573.6
Vit D (mcg/d)	4.6 ± 4.7	5.0 ± 2.8
Vit E (mg/d)	9.1 ± 7.6	7.5 ± 3.9
folate (mcg/d)	383.2 ± 154.7	502.2 ± 119.6
Ca (mg/d)	778.3 ± 355.0	1009.6 ± 537.0
Fe (mg/d)	16.7 ± 5.7	18.1 ± 5.1
Vegetable (servings/d)	4.2 ± 2.1	3.6 ± 1.9
Fruit (servings/d)	3.5 ± 1.9	3.9 ± 1.0
Cereal/Grain (servings/d)	2.7 ± 0.9	5.0 ± 1.7

Project #5, Table 3: Estimated intake of soy protein or lycopene based upon compliance records (first 38 men enrolled of an expected 40). The compliance with soy was excellent and the men demonstrated an ability to consume lycopene at a very high concentration through foods alone (no supplements)

	Soy Group Wks 0-4	**Tomato Group Wks 0-4**	**Tomato + Soy Group Wks 4-8**
	mean ± SD	mean ± SD	mean ± SD
Soy Protein Intake (g/d)	39 ± 1	0	38 ± 4
Lycopene Intake (mg/d)	0	43 ± 15	37 ± 16

Project #5, Table 4: IGF-I in the first 38 men enrolled of 40 expected. No changes in IGF-I were observed and it is unlikely that significant changes will be detected even with 2 additional men assigned to the trial.

	IGF-1 Week 0	**IGF-1 Week 4**	**IGF-1 Week 8**
	mean ± SD	mean ± SD	mean ± SD
Tomato Group (ng/mL)	161 ± 69	181 ± 75	175 ± 72
Soy Group (ng/mL)	171 ± 93	165 ± 89	184 ± 84

Project #5, Table 5. PSA changes in the first 38 men enrolled in the clinical trail. A total of 40 men are expected to be enrolled. This data should be viewed as preliminary until a complete statistical analysis of all 40 men has been completed. Final evaluation will include analysis at 4 weeks and 8 weeks of intervention.

	Tomato Group (4 wks tomato plus 4 wks combined) (n = 20)	**Soy Group (4 wks tomato plus 4 wks combined) (n = 18)**
	mean ± SD	mean ± SD
% with rising PSA prior to enrollment	100% (=20)	100% (n=18)
% with lower PSA at end of the 8 wk study than at enrollment	30% (n = 6/20)	50% (n = 9)
% with prolonged doubling time compared with pre-enrollment	60 % (n= 12/20)	50% (n = 9/14)*
* *Not enough data to determine pre-study doubling time in 4 men*		

BULLETED LIST OF KEY ACCOMPLISHMENTS (Project #5).

Accomplishments of the clinical trial (based upon preliminary interim analysis)

- Successfully completed recruitment of 40 patients.
- Established dietary intake score cards for food based lycopene consumption.
- Documented safety (lack of toxicity) for soy products at 80 mg/day isoflavones, lycopene at over 40 mg/day from tomato products, and for the combination consumed simultaneously.
- Observed no significant effect of soy, tomatoes, or both soy and tomato products on serum IGF-I.
- Inhibition of PSA rise (PSA velocity) by combining soy and tomato products.

REPORTABLE OUTCOMES.

Each of the above projects contributed to the training of the fellows supported by the training grant. The described reportable outcomes in each of the projects and has been or will be submitted for publication. This work in projects 1-4 was included to help demonstrate the training and accomplishments of the fellows and show that they contributed to significant research directly related to the clinical trail proposed in the SOW.

The clinical trial (Project 5), that was the basis of the original SOW will be terminated after the participation of 40 men (currently n=38). All of the additional outcomes will then be evaluated (blood carotenoid patterns, lycopene isomers, urinary soy isoflavones, blood isoflavones, hormone patterns, antioxidant biomarkers) and reported in a publication.

The benefits of the support included the training of Drs. Boileau, Carlton, and Wang. Dr Boileau was instrumental in developing the initial protocol for the clinical trial and establishing many of the analytical assays for the biological samples. He has taken a senior laboratory position at Procter and Gamble, Inc. where he will be developing dietary interventions for the prevention of prostate and other cancers using the dog model. As you may know the dog is the only species other than humans to develop spontaneous prostate cancer at a significant rate. He will continue to collaborate regarding data analysis and preparation of the publication from the clinical study. Dr. Carlton was briefly supported by the project and quickly applied for, and was rewarded, with a T32 postdoctoral training grant. He subsequently redirected his work away from dietary inhibitors of carcinogenesis to novel derivatives of celecoxib that target similar pathways as soy isoflavones. He has directed his current research to focus upon novel AKT signal transduction inhibitors for genitourinary malignancies, and based upon his data and that of others, the novel inhibitors synthesized at OSU have been accepted by NCI to be developed as part of the RAID program for GLP synthesis and preclinical toxicology testing prior to Phase I trails at OSU. Dr. Wang was the most recent individual supported by the DOD and he has been active in procurement and processing of biological samples from the clinical trail as well as the analysis of biomarkers. Dr. Wang is a co-investigator on an NCI-RO1 submitted in October 2004 entitled, "novel tomato-soy products for prostate cancer prevention", that is a direct outgrowth of the clinical project. We have developed a new soy-tomato juice drink based upon the safety demonstrated by our clinical trial (combination of soy and tomato was exceptionally well tolerated). This project if funded would support a randomized phase IIb clinical trial evaluating increasing dosages of soy-tomato juice in the neoadjuvant setting with the goal of evaluation distribution of phytochemicals to the prostate and effects of biomarkers.

CONCLUSIONS.

The studies from our laboratory group, including the efforts of Drs. Boileau, Carlton, and Wang during the last 4 years have contributed significantly to the knowledge in the area of diet, nutrition, and chemoprevention (see references below). The training and research experience for each of these outstanding young investigators has positioned each to be successful in their future careers. The clinical trial will be completed in the next few weeks and the analysis of all data should be done by spring 2005 and a manuscript prepared.

An interim analysis of the data shows that men will consume a combination of soy and tomatoes to provide a high intake of isoflavones (80 mg/day) and lycopene respectively (40 mg/day) without significant toxicity. Furthermore, preliminary analysis suggests that PSA velocity may be reduced in this cohort of men with progressing prostate cancer.

This work will impact future cancer prevention efforts. Based upon this data we have created a high-lycopene tomato juice enriched in soy isoflavones. This "nutriceutical" will provide a convenient method of providing these phytochemicals to patients in large scale clinical trials that will be designed to examine cancer prevention outcomes.

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APPENDIX.

1. IN PRESS. Liao Z, Wang S, Boileau TW, Erdman JW, Clinton SK. *Increased phospho-Akt is associated with loss of the androgen receptor during the progression of N-Methyl-N-Nitrosourea-induced prostate carcinogenesis in rats.* Prostate, 2005.

2. IN PREPARATION. Guo Y, Wang S, Hoot DR, Clinton SK. Genistein inhibits VEGF-mediated autocrine and paracrine angiogenesis network between prostate cancer cells and vascular endothelial cells.

3. Tomato and soy product survey.

4. Tomato product consumption log.

5. Soy product consumption log.

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Increased Phospho-AKT Is Associated With Loss of the Androgen Receptor During the Progression of *N*-Methyl-*N*-Nitrosourea-Induced Prostate Carcinogenesis in Rats

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BACKGROUND. Characterization of molecular events during *N*-methyl-*N*-nitrosourea (MNU)-induced rat prostate carcinogenesis enhances the utility of this model for the preclinical assessment of preventive strategies. Androgen independence is typical of advanced human prostate cancer and may occur through multiple mechanisms including the loss of androgen receptor (AR) expression and the activation of alternative signaling pathways.

METHODS. We examined the interrelationships between AR and p-AKT expression by immunohistochemical staining during MNU-androgen-induced prostate carcinogenesis in male Wistar-Unilever rats. Histone nuclear staining and image analysis was employed to assess parallel changes in chromatin and nuclear structure.

RESULTS. The percentage of AR positive nuclei decreased ($P < 0.01$) as carcinogenesis progressed: hyperplasia (92%), atypical hyperplasia (92%), well-differentiated adenocarcinoma (57%), moderately-differentiated adenocarcinoma (19%), and poorly-differentiated adenocarcinoma (10%). Conversely, p-AKT staining increased significantly during carcinogenesis. Sparse staining was observed in normal tissues (0.2% of epithelial area) and hyperplastic lesions (0.1%), while expression increased significantly ($P < 0.001$) in atypical hyperplasia (7.6%), well-differentiated adenocarcinoma (16.7%), moderately-differentiated adenocarcinoma (19.6%), and poorly-differentiated adenocarcinoma (17.4%). In parallel, nuclear morphometry revealed increased nuclear size, greater irregularity, and lower DNA compactness as cancers became more poorly differentiated.

CONCLUSIONS. In the MNU model, the progressive evolution of dominant tumor cell populations showing an increase in p-AKT in parallel with a decline in AR staining suggests that activation of AKT signaling may be one of several mechanisms contributing to androgen insensitivity during prostate cancer progression. Our observations mimic findings suggested by human studies and support the relevance of the MNU model in preclinical studies of preventive strategies. *Prostate 9999: 1–14, 2005.* © 2005 Wiley-Liss, Inc.

KEY WORDS: **prostate cancer; androgen receptor; p-AKT; nuclear morphometry; rats**

Abbreviations: MNU, N-methyl-N-nitrosourea; AR, androgen receptor; IHC, immunohistochemical staining; AIPC, androgen independent prostate cancer; p-AKT, phospho-AKT.

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INTRODUCTION

Novel chemopreventive strategies and dietary interventions are being characterized with the hope that they may reduce prostate cancer risk, but few can be tested in humans due to the expense of large long-term studies. Rodent models provide an opportunity for the preclinical assessment of the efficacy and toxicity of preventive strategies and may help prioritize interventions for future human translational studies. In addition, rodent models allow investigators to characterize biomarkers that can be subsequently employed as surrogate endpoints in short-term human studies that are necessary to define optimal dosing strategies for definitive human prevention studies of longer duration. The *N*-methyl-*N*-nitrosourea (MNU)-androgen-induced model of prostate carcinogenesis in rats has emerged as a valuable tool in this regard [1,2]. The model demonstrates a time-dependent appearance of histopathologic lesions encompassing the spectrum of prostate carcinogenesis from hyperplasia and dysplasia to invasive carcinoma [3,4]. Although few studies have characterized biomarkers in this model, the interrelationships between angiogenesis, proliferation, and apoptosis during MNU-induced prostate carcinogenesis shows a strong correlation with published human data [4]. The present study examines the expression of additional biomarkers that have been hypothesized to change during human prostate carcinogenesis.

Androgens are required for the growth, development, and function of the prostate in humans and laboratory rodents [5]. The early phases of prostate carcinogenesis are promoted by androgens, in part by stimulating proliferation and inhibiting apoptosis [3,6,7]. Indeed, the inhibition of testosterone metabolism by a 5-alpha reductase inhibitor (finasteride) was recently demonstrated as an effective strategy for prostate cancer chemoprevention in humans [8]. Castration or pharmacological interventions to deplete androgen activity is an effective palliative therapy for advanced prostate cancer [9]. However, androgen-independent prostate cancer (AIPC) emerges in men treated with anti-androgen therapy [10,11], a hallmark of incurable and lethal prostate cancer progression.

The multiple mechanisms whereby prostate cancer cells develop androgen independence are beginning to be elucidated [11–13]. Some mechanisms are associated with a loss of androgen receptor (AR) expression and may contribute to the heterogeneity of immunostaining for AR observed with increasing the Gleason score [14] compared to the typical homogeneous staining observed in normal prostate epithelium [15]. However, several mechanisms for the evolution of AIPC may occur in the presence of AR [11–13]. Overexpression of the AR resulting in "supersensitivity" to androgens is one possibility [13]. The activation of parallel or alternative survival signaling pathways, which bypass the dependence of the cell for androgens or AR-regulated gene expression is one hypothesis [11]. Accumulating evidence suggests that overexpression or constitutive activation of the phosphoinositol-3-kinase/AKT signaling pathway is one example of the phenomena. This may occur through inactivation of the phosphatase PTEN leading to accumulation of the active phosphorylated AKT [16–19]. The downstream effects of activated phospho-AKT (p-AKT) are apoptotic resistance and uncontrolled proliferation [17,18,20]. Indeed, studies with human prostate tissue suggest a loss of PTEN expression in advanced cancer [21] and increased AKT [22] or p-AKT with higher Gleason grade [23]. These observations from human tissues have not been carefully evaluated in rodent models of prostate carcinogenesis that serve as tools to assess preventive interventions.

The progression of prostate cancer is associated with genetic instability causing changes in gene expression profiles and tumor biology [24]. The detailed elucidation of critical molecular genetic events, such as changes in AR and p-AKT expression, was preceded by fundamental observations beginning in the mid 19th century by Virchow who described morphological changes in the nucleus that are characteristic of cancer [25,26]. It is hypothesized that DNA damage and aberrant gene expression in prostate carcinogenesis may, in part, be related to changes in nuclear matrix and structural components that may underlie the morphologic observations [27,28]. However, speculation remains concerning whether altered nuclear structure is a determinant of genetic instability or if the dynamic changes in chromatin organization and nuclear morphology is the result of DNA damage [28,29]. In addition, it has been a challenge to establish an objective and quantitative grading system for nuclear alterations that can be used to predict biological behavior and serve as a biomarker in experimental studies or as a prognostic factor in clinical specimens. Recent developments in digital imaging of histopathologic specimens and computerized image analysis provide an opportunity to reassess nuclear morphometry in an objective and quantitatively reproducible manner [30,31]. The present study describes the application of these tools for the assessment of nuclear morphometry in the rat MNU prostate carcinogenesis model and its relationship to changes in AR and p-AKT expression.

The studies reported herein examine the temporal interrelationships in AR and p-AKT expression in parallel with changes in nuclear morphometry during the progression of MNU androgen-induced prostate

carcinogenesis in Wistar-Unilever rats. These findings provide further details regarding critical events during prostate cancer progression in this clinically relevant model and may suggest targets for novel chemopreventive agents or dietary constituents. Furthermore, these observations provide valuable insight regarding the characterization and interpretation of biomarker expression and may prove to have application in studies evaluating novel interventions for prostate cancer prevention in humans.

MATERIALS AND METHODS

Animals and Diets

The tissues examined in this study are from male Wistar-Unilever rats (Harlan, Indianapolis, IN) composing the control group in a large study evaluating the ability of dietary lycopene, tomato products, and energy restriction to inhibit prostate carcinogenesis [2]. The control rats were fed an AIN-93G-based semipurified diet [32] stored in the dark at 4°C until fed. Rats were housed in a room with a 12 hr dark-light cycle and were allowed free access to water and the AIN-based semipurified diet [32]. Several male Wistar-Unilever rats without any treatment (no carcinogen or hormone manipulation) were also included for histological studies of normal prostate tissue.

Prostate Tumor Induction

Carcinogen and hormone exposure followed protocols previously described [1,2,4,33]. At 6 weeks of age, each rat received daily i.p. injections of cyproterone acetate (Sigma Chemical, St. Louis, MO) for 21 consecutive days. One day following the last dose of cyproterone acetate, rats received a daily s.c. injection of 100 mg testosterone proprionate (Sigma Chemical)/kg body weight in 0.5 ml soybean oil for 3 days. One day after the last testosterone proprionate injection all rats received a single i.v. dose (50 mg/kg body weight) of MNU (Ashe Stevens, Inc., Detroit, MI) via the tail vein under metophane anesthesia. One week after MNU administration, rats received continuous exposure to testosterone via 2 subcutaneous implants (1.02 mm ID × 2.16 mm OD × 2.54 cm length silastic laboratory tubing; Dow Corning, Midland, MI) each containing 30 mg crystalline testosterone (Sigma Chemical). All animal procedures were approved by the University of Illinois Laboratory Animal Care Advisory Committee.

Necropsy

Rats were monitored daily throughout the experiment. Those surviving until the planned necropsy at 64 wks, or those exhibiting signs or symptoms of prostate cancer at earlier time points, were euthanized by CO_2 inhalation. Immediately after sacrifice, the prostate and seminal vesicles were removed en bloc and placed in 10% neutral buffered formalin. The tissues were grossly examined and dissected into seven sections for embedding unless extensive cancer involvement precluded dissection: (1) bladder, (2) right ventral lobe, (3) left ventral lobe, (4) right dorsolateral lobe, (5) left dorsolateral lobe, (6) right seminal vesicle and coagulating gland, and (7) left seminal vesicle and coagulating gland. Light microscopy of H&E stained slides was employed for histopathologic classification of lesions according to criteria previously defined [1,2,4,34].

Immunostaining

Rabbit anti-human AR antibody (N-20) against the N-terminus of AR, reported to cross-react with rat AR was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Sections were prepared from embedded blocks, subjected to deparaffinization, rehydration and washing, followed by heating at 100°C in 100 mM citrate buffer (pH 6.0) for 15 min and allowed to cool to room temperature. The subsequent steps were completed in an OptiMax Automated Cell Staining System (BioGenex, San Ramon, CA) at room temperature using the Dako EnVision+ kit (Dako Corp., Carpinteria, CA). Endogenous peroxidase and non-specific binding were blocked by a H_2O_2 blocking solution (Dako Corp.), followed by a 30 min incubation with the primary antibody at 1:300 dilution. After three washes with PBS, sections were incubated with a polymer of anti-rabbit avidin-biotin-horseradish peroxidase for 30 min. Color was developed with 10 min incubation with diaminobenzidine (DAB) chromogen solution. Slides were counterstained with hematoxylin for 2 min and mounted. Negative controls include rabbit serum and omission of primary antibody. We employed sections known to be positive for AR and negative for AR in each assay. Cell blocks prepared from the human LNCaP prostate cancer cell line and PC3 cell line serve as positive and negative controls.

In order to assess the specificity of the AR antibody we completed a Western blot using the LNCaP cell line. Whole cell lysates were prepared according to a protocol from Santa Cruz Biotechnology. Protein content of each sample was determined by the Bio-Rad assay (Bio-Rad, Hercules, CA) and 100 μg protein per lane was loaded onto precast 12% SDS–PAGE gels (Novex, San Diego, CA). Gels were electrophoresed and then transferred onto nitrocellulose membranes (Bio-Rad) according to the manufacturers' instructions. The membranes were blocked for 1 hr at room temperature in 5% non-fat dry milk dissolved in TBST (20 mM Tris-HCl pH 8.0, 137 mM NaCl, and

0.1% Tween 20) and subsequently washed three times (10 min per wash) with TBST. The primary antibody (N-20 at 1:1000) for AR (Santa Cruz Biotechnology) was applied and incubated for 1 hr at room temperature. The membranes were washed three times (10 min per wash) with TBST. Anti-rabbit HRP secondary antibody (Amersham, Piscataway, NJ) at 1:10,000 dilution was incubated for 1 hr at room temperature. After three washings, membranes were treated with luminol reagent for detection (ECL systems, Santa Cruz Biotechnology).

The rabbit anti-p-AKT (Cell Signaling Technology, Inc., Beverly, MA) immunostaining methods were identical to those described for AR with the exception that the primary antibody was incubated overnight at 4°C at 1:300 dilution. The assay was optimized in preliminary studies using a range of antibody dilutions and non-immune rabbit serum in place of primary antibody was employed as the negative control. The mouse anti-histone H1 (AE-4) was purchased from Santa Cruz Biotechnology. Murine non-specific isotype antibody was used as the negative control. The immunostaining methods were as described for AR with the exception that the primary antibody was incubated for 30 min at room temperature with 1:500 dilution.

Image Analysis of Immunostained Sections

Images from H&E, AR, p-AKT, and histone H1 IHC staining were captured at 400× magnification by a high resolution digital camera (Spot RT, Diagnostic Instrument, Inc., Sterling Heights, MI) using bright field microscopy (Nikon ECLIPSE E 800, Tokyo, Japan) and transmitted to an image analysis workstation (6500 Pentium III WorkStation, Dell Computer Corp, Round Rock, TX). Specific outcomes were analyzed using image analysis software (Image-Pro Plus 4.1, Media Cybernetics, Silver Spring, MD).

For AR staining of normal rat prostate sections, image analysis involved the following steps. We chose to separate the images into three compartments: epithelial, nonepithelial (primarily stoma and vascular compartments), and lumens/secretions. Poorly stained areas (edges) or folds were removed manually from the image and other nonspecific structural artifacts were eliminated by setting the size and shape filters. The nuclei in the epithelial and stromal compartments were evaluated separately for AR staining. Nuclei were then segmented into two classes of binary color images: red for AR positive nuclei and yellow for AR negative nuclei. The percentage of positive nuclei was then calculated based on the following formula: labeling index $(\%) = L/(L + C) \times 100$, where L = labeled cells (red) and C = counterstained, unlabeled cells (yellow). Similar techniques were employed for the quantitative evaluation of various lesions (hyperplasia, atypical hyperplasia, and carcinomas of varying differentiation). We digitally eliminated stroma and lumens/secretions from the image and quantitated the percentage of positive nuclei in the epithelial compartment.

The free-drawing tool in Image-Pro Plus was used to differentiate the epithelial compartment from the stroma/glandular lumens and artifacts. To perform the p-AKT analysis, we segmented the brown color from the background and calculated the percentage of area staining positive using a similar approach as described for AR staining.

Sections were stained for histone H1 for nuclear morphometry and chromatin density analysis. Histone H1 positive, non-overlapping, and intact nuclei were identified by the image analysis software after applying various filter sizes to eliminate nonspecific objects. The system was calibrated with a micrometer before image analysis was performed. Each image was captured using a 40× objective with a numerical aperture of 0.65 using a high-resolution 24-bit digital camera (1,520 × 1,080 pixels). We obtained quantitative data on several parameters: (a) nuclear area (μm^2), (b) eccentricity (the ratio of the major axis to minor axis), (c) sphericity (the ratio of minimum to maximum radius measured from the nuclear centroid), (d) chromatin compactness (mean absorbance of the nucleus stained by histone H1 IHC), and (e) mean, minimal, and maximal diameters.

Statistical Analysis

All quantitative data was analyzed by one way analysis-of-variance (ANOVA) after testing for normal distribution and log transformation if necessary. A significant ANOVA ($P < 0.05$) is then followed by Fisher's protected least-significant difference to assess two-sided pairwise comparisons among histological grades or different anatomical locations of normal accessory sex glands (StatView 5.01, SAS Institute, Inc., Cary, NC). Data are reported as mean ± standard deviation (SD).

RESULTS

Sensitivity and Specificity of the AR Antibody

The sensitivity and specificity of the AR antibody (N-20) was examined by Western blot and IHC. Western blot demonstrated a 110 kDa protein migrating at the known AR molecular weight (Fig. 1A). Immunohistochemical analysis using the AR antibody revealed strong nuclear staining in the LNCaP cell line (Fig. 1B), and in normal rat testis, prostates, and ovary (data not shown) as expected. Moderate staining was observed in the normal rat uterus and brain with no

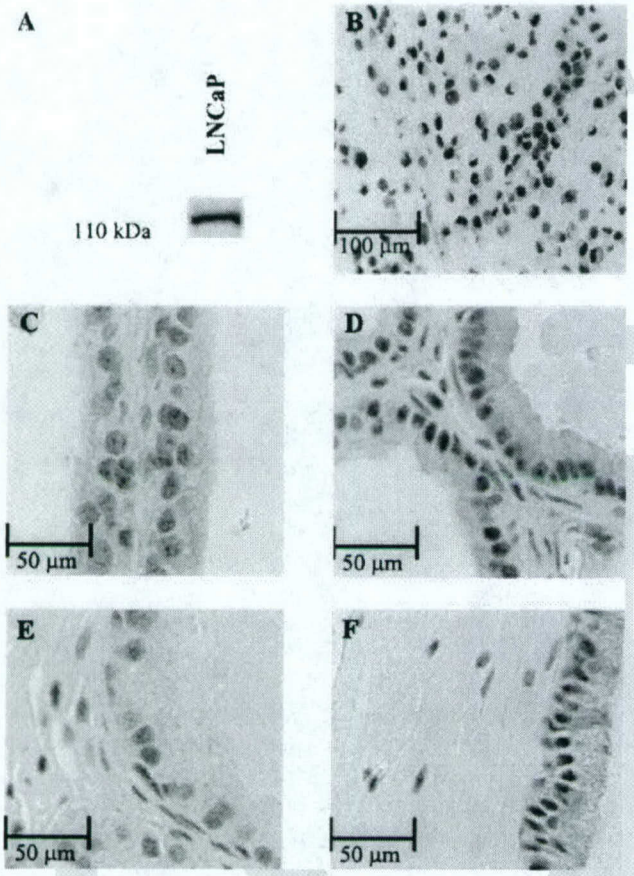


Fig. 1. Evaluation[Q4] of anti-androgen receptor (N-20) antibody (**A–B**) and a comparison of immunostaining for AR in the different lobes of the normal rat prostate (**C–D**). A: A representative Western blot demonstrating that the antibody binds a single 110 kDa protein in a cell lysate from the AR-positive human LNCaP prostate cancer cell line. B: Immunostaining of LNCaP cells demonstrates strong nuclear staining. C: Ventral lobe; (D) dorsolateral lobe; (**E**) coagulation gland; and (**F**) seminal vesicle. Overall, the ventral lobe showed lower intensity of AR staining than dorsolateral lobe, coagulation gland, and seminal vesicle (C, F).

staining in liver, kidney, pancreas, intestine, and spleen (data not shown) or the AR negative PC-3 cell line. Thus, the N-20 antibody appeared to exhibit sensitivity and specificity characteristics required for the quantitation of AR expression in rat tissues.

Nuclear Androgen Receptor Staining Within the Non-malignant Prostate

AR staining was localized in nuclei of cells in the stroma and epithelium of accessory sex glands of rats that show no evidence of cancer (Fig. 1C,F and Table I). Overall, the percentage of cells showing nuclear AR staining was greater in the epithelial compartment than in the surrounding stromal compartment (Table I). In addition, significant differences in nuclear AR staining were observed among the various structures within the accessory sex gland complex ($P < 0.05$). AR staining of the epithelium (mean ± SD) was greater ($P < 0.05$) in the seminal vesicle (89% ± 9%), coagulation gland (89% ± 17%), and dorsolateral lobes (80% ± 27%), compared to the ventral lobes (46% ± 278%) (Fig. 1 and Table I). Although not quantitated, the intensity of AR staining also appeared to be lower in ventral lobe than in dorsolateral lobe, coagulation gland, and seminal vesicle (Fig. 1C,F). Interestingly, stromal nuclear AR staining (mean ± SD) was greatest ($P < 0.05$) in the seminal vesicle (64% ± 10%), intermediate in the coagulation gland (48% ± 5%), and lower in the dorsal (14% ± 5%) and ventral (6% ± 5%) lobes of the prostate (Table I).

Androgen Receptor Nuclear Staining During Prostate Carcinogenesis

All non-cancerous prostate tissues as well as lesions defined as hyperplasia and atypical hyperplasia showed AR staining (Table II). As cancer progressed, the percentage of prostate lesions with positive AR staining decreased. Ninety percent of prostate tissues

TABLE I. Androgen Receptor Immunostaining of Normal Epithelial and Stromal Cells in Specific Anatomical Locations of Rat Prostate Tissue*

	Dorsal lobe	Ventral lobe	Seminal vesicle	Anterior lobe/ coagulation gland
Samples (n)	29	12	25	23
Epithelial nuclei (%)	80 ± 27^a	46 ± 28^b	89 ± 9^a	89 ± 17^a
Stromal nuclei (%)	14 ± 5^a	6 ± 5^a	64 ± 10^b	48 ± 5^c

*Samples evaluated for AR expression were selected from rats not showing histologic evidence of cancer within the anatomical structure. The data represents the percentage of nuclei stained positive within the epithelial or stromal compartment of a 400× high power field (mean ± SD). Means in each row with different superscripts indicate a significant difference between other anatomical sites, $P < 0.05$, by ANOVA and pair-wise comparisons.

Fig. 1.

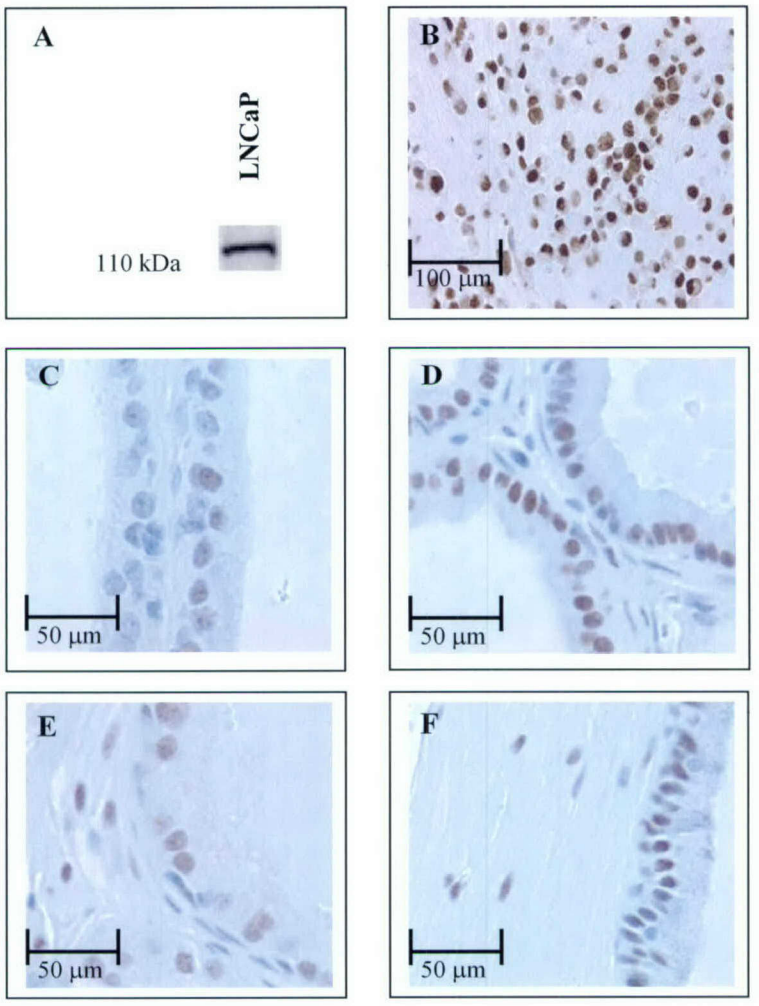


TABLE II. The Percentage of Rat ProstateTissues and Lesions With Positive AR Staining and the % of Cells Staining Positive Within the AR Positive Section (% of Positive Nuclei in a 400× Field)*

				Adenocarcinoma		
	Normal	Hyperplasia	Atypical hyperplasia	Well differentiated	Moderately differentiated	Poorly differentiated
Rats (n)	57	28	23	22	8	46
Tissue samples staining positive (%)	100	100	100	90	50	28
Percentage of AR positive cells (%)	80 ± 25^a	92 ± 6^b	92 ± 9^b	57 ± 30^c	19 ± 18^d	10 ± 18^d

*Means with different superscripts indicate a statistically significant difference, $P < 0.01$ by ANOVA and pairwise comparison.

with well-differentiated adenocarcinoma, 50% with moderately-differentiated adenocarcinoma and 28% with poorly-differentiated adenocarcinoma showed AR staining (Table II). Among these positively stained prostate tissues, the percentage of cells with nuclear AR staining was slightly higher ($P < 0.01$) in hyperplastic lesions ($92\% \pm 6\%$) and atypical hyperplasia ($92\% \pm 9\%$) compared to the adjacent histologically normal appearing glands ($80\% \pm 25\%$) (Table II). As prostate carcinogenesis progressed, the percentage of AR positive nuclei significantly declined (Fig. 2). We observed $57\% \pm 30\%$ of cells staining in well-differentiated adenocarcinoma, $19\% \pm 18\%$ in moderately-differentiated adenocarcinoma, and only $10\% \pm 18\%$ in poorly-differentiated adenocarcinoma ($P < 0.01$ compared to hyperplastic lesions). We occasionally observed lesions exhibiting spatial heterogeneity in AR expression with areas showing strong staining adjacent to areas of weak staining (Fig. 3). It is tempting to hypothesize that the lesion may contain a subpopulation of cells undergoing evolution to an androgen independent state with the loss of AR expression.

Activation of p-AKT Expression During Prostate Carcinogenesis

p-AKT staining was typically localized to the cytoplasm and cell membrane in prostate lesions (Fig. 4, Table III). In nonmalignant rat prostate tissues, only $0.2\% \pm 0.3\%$ (mean ± SD) of the epithelial compartment showed positive staining for p-AKT and was similar to that observed for low-grade hyperplastic prostate lesions ($0.1\% \pm 0.1\%$). p-AKT staining increased significantly ($P < 0.001$ vs. normal and hyperplasia) in atypical hyperplasia ($7.6\% \pm 9.7\%$), and further increased in the lesions identified as carcinoma ($P < 0.001$ vs. atypical hyperplasia) with a range of 16.7%–19.6% of the lesion "area" stained positive with no significant difference between carcinomas based upon differentiation. A stepwise increment in staining during carcinogenesis can be proposed based upon these observations. The first occurs between benign hyperplasia and atypical hyperplasia (>35 fold increase) ($P < 0.001$ compared with normal or hyperplastic prostate tissues) and from atypical hyperplasia to cancer (2 fold increase) ($P < 0.001$ compared with atypical hyperplasia) (Table III).

Nuclear Morphometry During Prostate Carcinogenesis

All nuclei in non-necrotic areas of histologic sections exhibited histone H1 staining. The nuclei were easily and rapidly separated from the background by image analysis (Fig. 2). Nuclear area was found to predictably change with prostate carcinogenesis. The mean nuclear area (Table IV, average size of individual nuclei) was similar in normal prostate epithelium ($23.0 \pm 3.0\ \mu m^2$) and hyperplastic lesions ($23.6 \pm 4.6\ \mu m^2$). In comparison, a significant 17% increase in mean nuclear diameter was observed for atypical hyperplastic lesions ($P < 0.01$, $27.3 \pm 5.0\ \mu m^2$) and a 48% increase for well-differentiated carcinoma ($P < 0.01$, $34.4 \pm 4.6\ \mu m^2$). A further significant increase in nuclear size was observed for moderately-differentiated adenocarcinoma ($45.6 \pm 6.2\ \mu m^2$) compared to low grade lesions ($P < 0.01$) and poorly-differentiated adenocarcinoma ($48.7 \pm 9.8\ \mu m^2$). These findings were consistent with other measurements of nuclear size such as mean, minimal, and maximal diameters (Table IV). The shape of the nucleus can be objectively assessed by a measurement of eccentricity or sphericity (Table IV). Eccentricity is the ratio of the major axis to minor axis. Sphericity is the ratio of the minimum radius to maximum radius measured from the centroid of the nucleus. The values of both parameters in poorly-differentiated prostate carcinomas were significantly different compared to normal epithelium, hyperplastic lesions, and low grade carcinoma, $P < 0.01$ (Table IV).

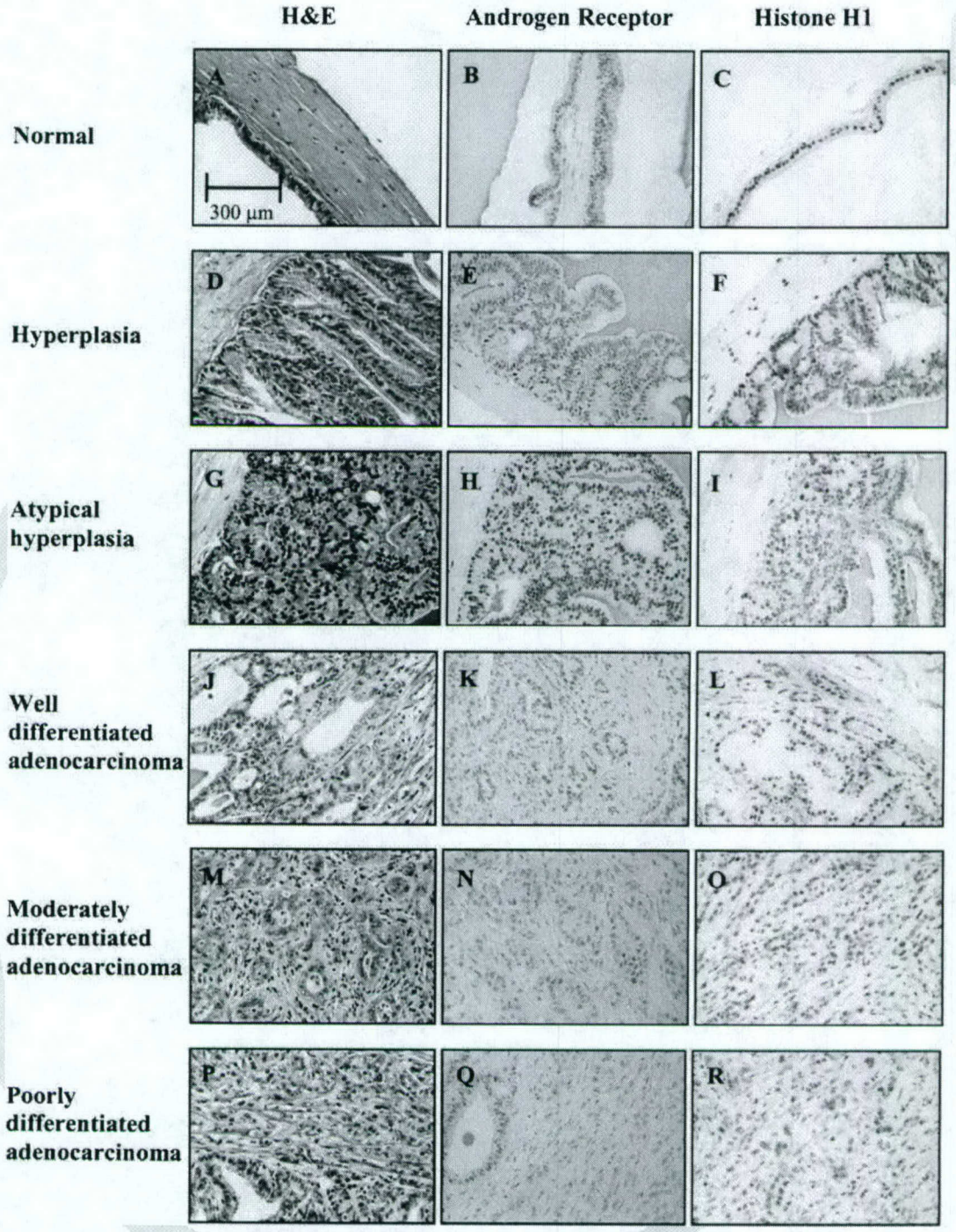
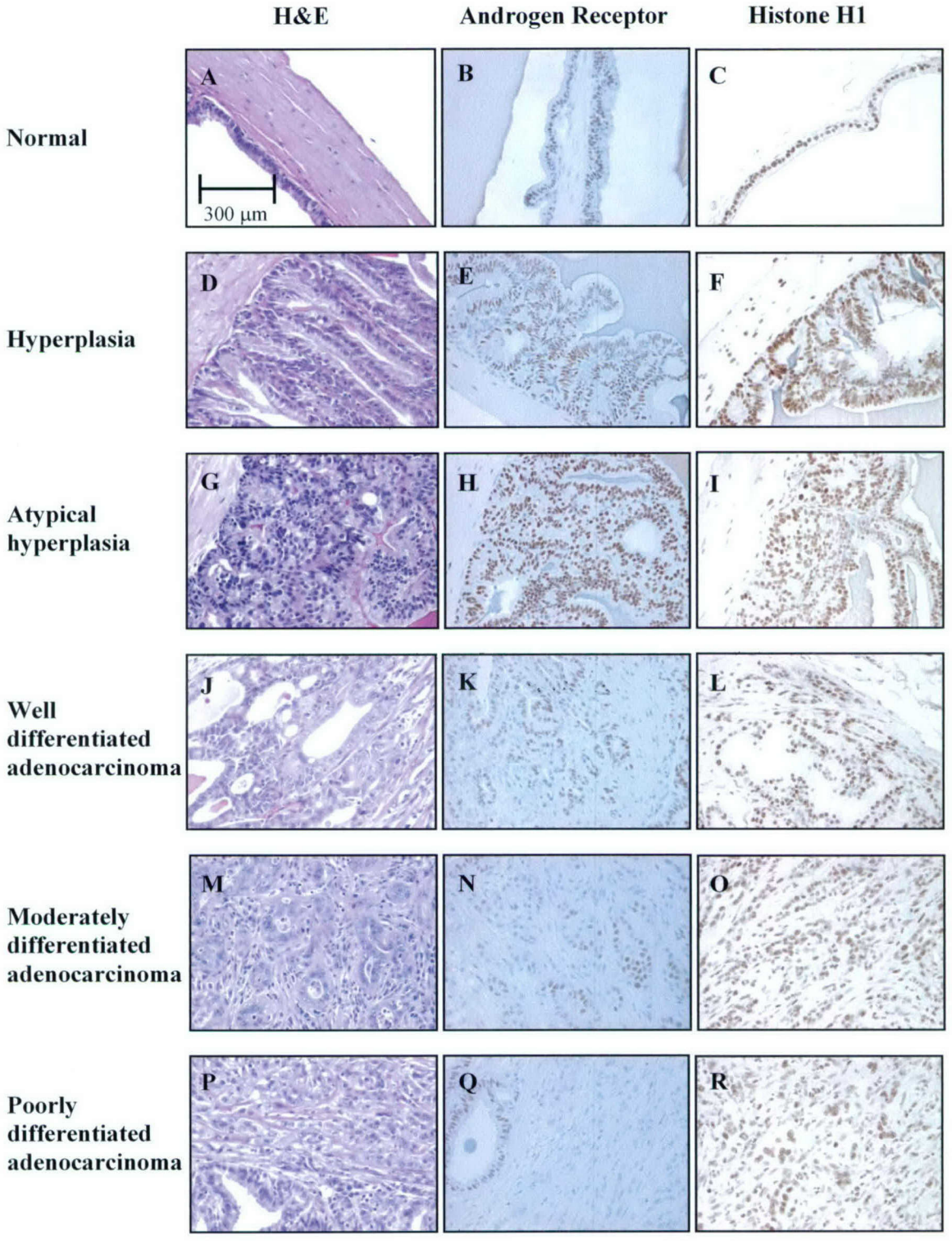


Fig. 2. Histopathology and immunohistochemical staining for AR and histone H1 in prostate tissue of rats treated with the carcinogen NMU and testosterone. The first column shows H&E staining of representative tissue ranging from normal epithelium to poorly-differentiated adenocarcinoma. The second column shows a decrease in AR IHC staining as cancer progresses. The third column shows histone H1 nuclear staining that becomes less intense in more aggressive cancer. **A**, **B**, and **C**: Normal epithelium; (**D**, **E**, and **F**) hyperplasial epithelium; (**G**, **H**, and **I**) atypical hyperplasia; (**J**, **K**, and **L**) well-differentiated adenocarcinoma; (**M**, **N**, and **O**) moderately-differentiated adenocarcinoma; and (**P**, **Q**, and **R**) poorly-differentiated adenocarcinoma.

Fig. 2.


H&E
Androgen Receptor
Histone H1
Normal
Hyperplasia
Atypical hyperplasia
Well differentiated adenocarcinoma
Moderately differentiated adenocarcinoma
Poorly differentiated adenocarcinoma
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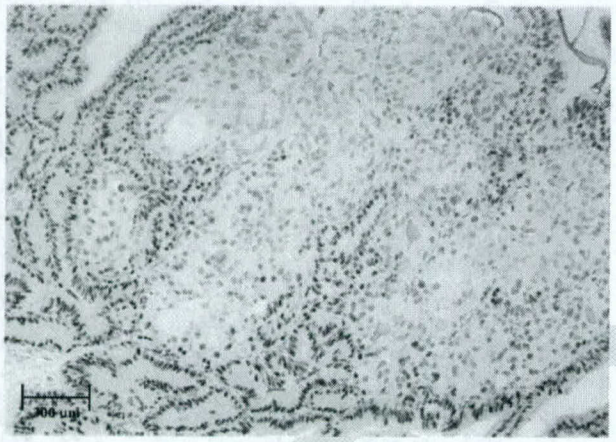


Fig. 3. AR expression in a representative lesion classified as atypical hyperplasia. Loss of AR nuclear staining is seen in the central portion of this lesion as compared to the peripheral area suggesting the evolution of the subpopulation exhibiting the loss of AR expression (200×).

Histone H1 staining also provides an assessment of DNA compactness as a measure of chromatin density. We observed the compactness of histone staining to be similar among normal epithelium, hyperplastic lesions, and well-differentiated carcinoma, but significantly reduced ($P < 0.01$) for moderately-differentiated carcinoma and further reduced for poorly-differentiated lesions (Table IV).

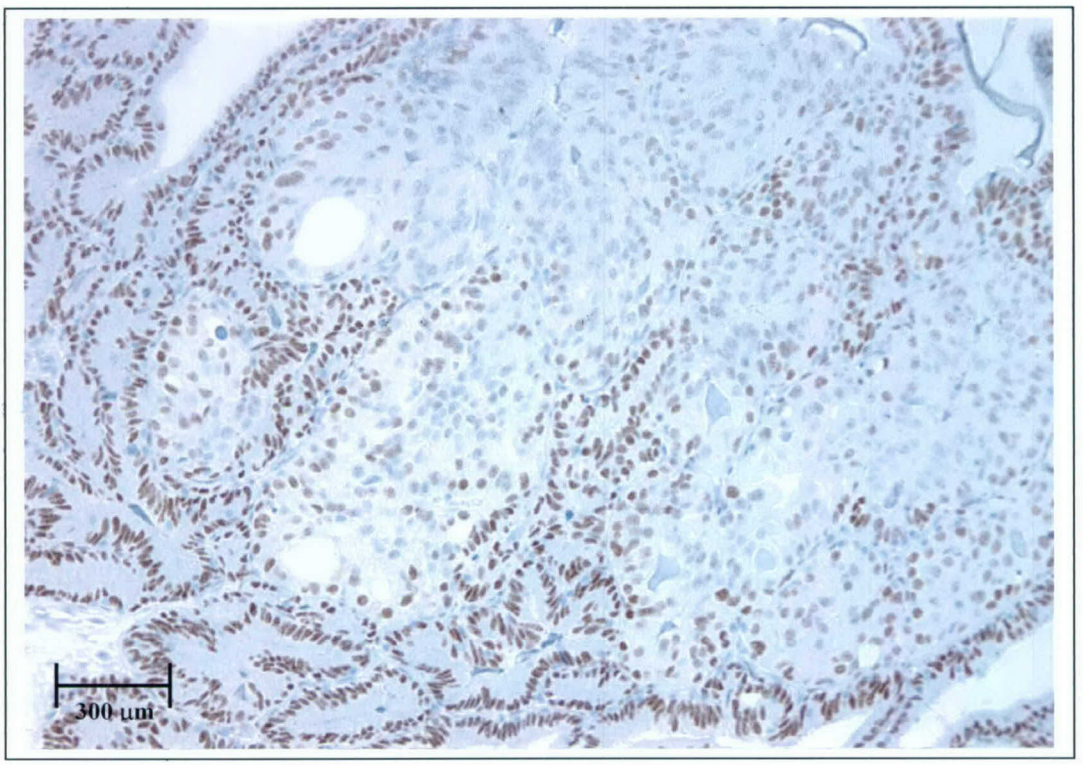
DISCUSSION

Animal models of prostate cancer are useful for elucidating mechanisms of carcinogenesis, for the evaluation of hypothesized environmental risk factors, and for the characterization of chemopreventive or dietary interventions that may reduce the incidence of disease. It is unlikely that any one model will perfectly mimic all the features of human prostate carcinogenesis. However, the MNU-testosterone-induced rat model has emerged as a valuable tool in studying prostate carcinogenesis [1,2,34] and investigators are beginning to validate biomarkers in this model and characterize their relevance to human disease [4]. The present studies document a loss of nuclear AR immunostaining in parallel with an increase in p-AKT and changes in nuclear architecture and chromatin density. Each of these changes in the MNU-model mimics findings from studies in human prostate tissues and further supports the value of this system for elucidating mechanisms of carcinogenesis relevant to the human disease and its prevention.

Androgens are critical to normal prostate growth, differentiation, and function while dysregulation of the androgen signaling pathway is a key feature of human prostate cancer progression culminating in an androgen independent lethal syndrome [11]. Thus, we considered it a high priority to document the changes in expression of nuclear AR during prostate carcinogenesis in the MNU model. We first focused upon staining in the different anatomical locations within the non-cancerous rat prostate gland. Our studies show much greater AR staining of the dorsolateral lobes, anterior prostate, and seminal vesicle, ranging from 80% to 89% of cells, than in the ventral lobe (46% of cells). Interestingly, this parallels the risk of developing cancer in the various anatomical regions following combined MNU-androgen exposure. Different rat strains show unique sensitivity and anatomical patterns of prostate and seminal vesicle carcinogenesis in response to MNU and androgens [3,35–37]. In the Wistar-Unilever rats employed in this study, cancer in the ventral lobe of the prostate is rare, while cancers in the dorsolateral lobe/anterior prostate are predominant with an additional 10%–30% of seminal vesicles showing lesions [38]. The molecular mechanisms that may underlie these observations are worthy of exploration and may provide insight into the importance of AR expression in the epithelium and sensitivity to carcinogens. It appears that testosterone is essential and indeed enhances early steps in the carcinogenesis cascade in the rat. Wistar-Unilever rats show no premalignant and cancerous prostate lesions under normal endocrine conditions, but approximately 5% of rats show lesions with additional testosterone treatment alone and a 20% incidence of lesions following MNU with physiologic androgen status, while over 60% will demonstrate lesions with both MNU and supplemental testosterone [1]. Thus, androgen signaling appears to establish a state where sensitivity to DNA damage by exogenous or endogenous mutagens is enhanced during the very early steps in the carcinogenesis process.

We observed a loss of AR nuclear staining during prostate carcinogenesis, as determined by both the percentage of lesions showing staining and the intensity of staining, with the progression to a more poorly-differentiated phenotype. The decline in AR immunostaining during prostate carcinogenesis has been suggested by human studies [39–44]. The decline of AR staining is an indicator that androgen signaling is profoundly altered in this model and suggests the evolution of an androgen insensitive tumor. However, the functional significance of this observation must be demonstrated in future studies, perhaps by transplantation of advanced MNU-induced prostate cancers, or cell lines derived from these tumors [45], into syngeneic rats or nude mice followed by treatment with castration or anti-androgens [45]. Although the loss of AR staining is probably a specific indicator of major defects

Fig. 3.



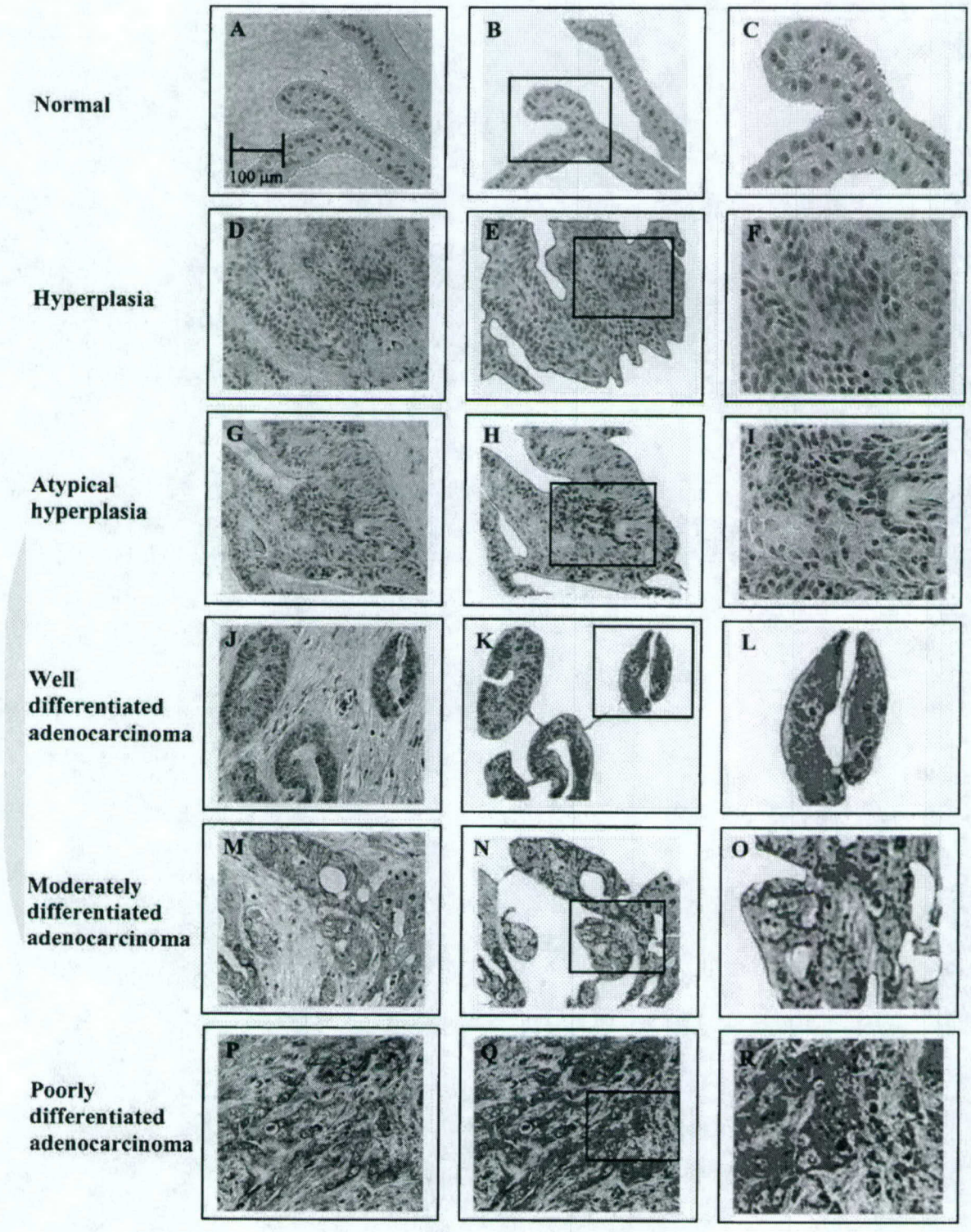
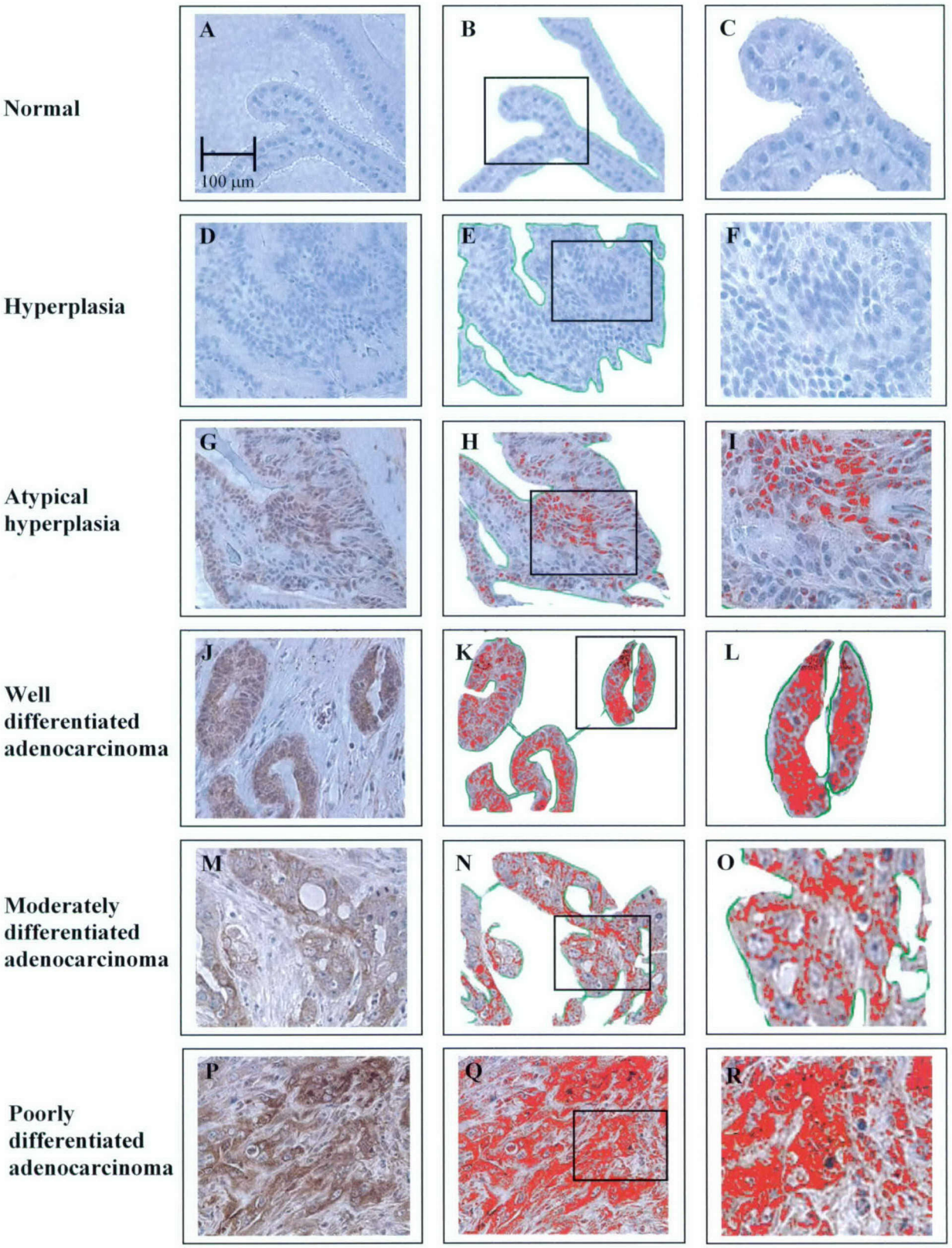


Fig. 4. Immunohistochemical staining and processed images for p-AKT in prostate tissue of rats treated with NMU and testosterone. The first column shows that the p-AKT staining. The second column shows the corresponding images processed by image analysis software. The images in the third column illustrate a higher magnification of the corresponding area from the second column. The stroma and normal glandular compartment were excluded with free drawing. Normal and hyperplasial epithelium showed no cytoplasmic staining in most of the rat prostate tissues. **A**, **B**, and **C**: Normal epithelium; (**D**, **E**, and **F**) hyperplasial epithelium; (**G**, **H**, and **I**) atypical hyperplasia; (**J**, **K**, and **L**) well-differentiated adenocarcinoma; (**M**, **N**, and **O**) moderately-differentiated adenocarcinoma; and (**P**, **Q**, and **R**) poorly-differentiated adenocarcinoma.

Fig. 4.


Normal
Hyperplasia
Atypical hyperplasia
Well differentiated adenocarcinoma
Moderately differentiated adenocarcinoma
Poorly differentiated adenocarcinoma
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100 μm

TABLE III. Immunohistochemical Staining for p-AKT Within Prostate Lesions (Percentage of Area of Positive Cells in a 400× Field) From RatsTreated With *N*-methyl-*N*-nitrosourea and Testosterone (Mean ± SD)*

				Adenocarcinoma		
	Epithelium	Hyperplasic epithelium	Atypical hyperplasia	Well differentiated	Moderately differentiated	Poorly differentiated
Rats (n)	24	17	16	9	13	24
Positivity (% of area)	0.2 ± 0.3^a	0.1 ± 0.1^a	7.6 ± 9.7^b	16.7 ± 14.7^c	19.6 ± 9.7^c	17.4 ± 12.8^c

*Means with different superscripts indicate a statistically significant difference, $P < 0.01$ by ANOVA and pairwise comparison.

in the AR signaling pathway, the presence of AR may not be a precise indicator of androgen responsiveness. For example, mutant AR receptors that retain the epitope targeted by the antibody may continue to stain positive but exhibit dysfunctional properties [11]. In addition, mutations in signaling pathways downstream from the AR or parallel pathways may cause functional androgen insensitivity while staining of the AR remains positive [11]. However, the profound changes in AR staining as prostate cancer progresses from early hyperplasic and dysplastic lesions to poorly-differentiated cancer in the MNU model suggest that the loss of AR staining is contributing to prostate cancer autonomy.

Our observations support the concept that the development of androgen independence is an inherent feature of progressive prostate carcinogenesis and does not require androgen deprivation to provide a selective evolutionary advantage for androgen-independent clones. Indeed supplemental androgens are continuously present in the MNU model. The concept that "spontaneous" mutations in androgen regulated pathways, including mutations in the AR, are universal features of prostate carcinogenesis is further supported by some elegant studies from the TRAMP model [46]. "Spontaneous" emergence of androgen independent prostate cancer occurs in parallel with the development of distinct classes of AR variants, with 15 unique somatic mutations in AR identified among cancers from only 8 mice [46]. Our work and these findings, indicate that somatic mutations in the *AR* gene, some of which may cause loss of immunostaining, occur spontaneously and frequently during carcinogenesis, probably in parallel with damage to cellular processes enhancing genetic instability, such as failure of DNA repair systems [47]. Thus, the MNU and TRAMP

TABLE IV. Changes in Nuclear Morphometry During MNU-testosterome Induced Prostate Carcinogenesis in Rats (Mean ± SD)*

				Adenocarcinoma		
	Normal epithelium	Hyperplasic epithelium	Atypical hyperplasia	Well differentiated	Moderately differentiated	Poorly differentiated
Rats evaluated	16	10	5	4	16	27
Total nuclei used	3,381	4,719	1,808	560	1,095	2,160
Mean nuclear area (μm^2)	23.0 ± 3.0^a	23.6 ± 4.6^a	27.3 ± 5.0^b	34.4 ± 4.6^c	45.6 ± 6.2^d	48.7 ± 9.8^d
Mean diameter (μm)	5.1 ± 0.3^a	5.2 ± 0.5^b	5.5 ± 0.5^c	6.2 ± 0.5^d	7.2 ± 0.5^c	7.4 ± 0.8^f
Minimal diameter (μm)	4.1 ± 0.4^a	4.1 ± 0.5^a	4.5 ± 0.6^b	5.0 ± 0.3^c	5.6 ± 0.4^d	5.6 ± 0.5^d
Maximal diameter (μm)	6.0 ± 0.4^a	6.2 ± 0.5^b	6.6 ± 0.5^c	7.4 ± 0.7^d	8.9 ± 0.6^e	9.4 ± 1.2^f
Eccentricity	1.44 ± 0.13^a	1.49 ± 0.12^b	1.44 ± 0.13^a	1.38 ± 0.08^a	1.51 ± 0.08^b	1.56 ± 0.07^c
Sphericity	0.55 ± 0.06^a	0.54 ± 0.07^a	0.56 ± 0.08^a	0.59 ± 0.04^b	0.54 ± 0.04^a	0.49 ± 0.04^c
Compactness of histone (OD) staining	0.22 ± 0.05^a	0.24 ± 0.06^a	0.21 ± 0.06^a	0.27 ± 0.11^a	0.19 ± 0.05^b	0.15 ± 0.06^c

Eccentricity is the ratio of the major axis to minor axis of the best-fit ellipse.
Sphericity is the ratio of the minimum radius to maximum radius measured from the centroid of the nucleus.
The compactness of histone staining is the mean absorbance of the nuclei stained with by IHC using anti-histone H1 antibody.
*Means within a row having different superscripts indicates a statistically significant difference, $P < 0.01$, by ANOVA and pairwise comparison. Data are pooled mean ± SD.

rodent models may be useful for the dissection of mechanisms involved in progression from the androgen-responsive to androgen-unresponsive states.

The loss of dependence upon exogenous growth factors and hormones in the tumor environment is one of the essential features of carcinogenesis [24]. This characteristic is, in part, achieved by mutations in intracellular signaling pathway that constitutively activate survival/anti-apoptotic and proliferative pathways. AKT is a critical protein in one signaling pathway that appears to be frequently activated in prostate carcinogenesis. Our data with the MNU model in rats shows a dramatic increase in p-AKT during the transition to dysplastic lesions with a further increase in carcinomas. Evidence for the importance of the AKT pathway in normal and malignant prostate cells is derived from several lines of evidence. Our laboratory and other groups have shown that AKT is involved in the signaling of IGF-I and other proliferative and survival factors for prostate cancer cells [19,20,48,49]. Prostate restricted AKT kinase transgenic mice demonstrate overexpression of activated AKT in the ventral prostate and spontaneously develop localized lesions histologically similar to prostatic intraepithelial neoplasia in humans [50]. The p-AKT is increased in human prostate cancer [51] and stronger staining is detected in lesions with higher Gleason scores [23]. The potential mechanisms underlying increased p-AKT staining are multiple. The phosphorylation of AKT is indirectly regulated by PTEN through PI3K. PTEN mutations are common in prostate cancer [52,53] and leads to the dysregulation of PI3K signaling and constitutive activation of AKT [49]. The activation of AKT may vary in different cancer cells. In colon cancer cells, AKT expression is up-regulated by the activation of IGF-1 receptor [54]; while overexpression of the ErbB2 receptor tyrosine kinase in breast cancer cells is correlated with the activation of AKT [55]. The loss of AR in parallel with increased p-AKT suggests that the two phenomena may be interrelated. Indeed, the constitutive activation of AKT pathways may mimic many of the activities of androgen regulated pathways thereby contributing to androgen independence [11]. AKT has been shown to be an essential cofactor for Her-2/neu induced AR signaling and androgen-independent survival and growth of human LNCaP of prostate cancer cells in vitro [56]. Overall, the robust over expression of p-AKT in the rat MNU model of prostate carcinogenesis is consistent with the little data available derived from human and in vitro observations and suggests that this phenomena may be a target for novel therapies [57] or preventive strategies [20].

Pienta and Coffey [28] in a seminal review, integrated data from divergent laboratory and clinical studies and crystallized a concept that the disruption of chromatin structure and nuclear architecture, perhaps contributing to alterations in gene expression and genomic instability, is an essential component of prostate carcinogenesis. Nuclear pleomorphism has been recognized in prostate cancer, which integrates the pathologists' subjective impression of changes in nuclear size and shape, chromatin texture, and the ratio of nuclear area to cytoplasmic area. Technology is now available for investigators to obtain quantitative data regarding many of these parameters. Histone H1 nuclear staining by IHC in conjunction with digital image analysis was employed as a means to quantitatively assess global changes in nuclear and chromatin structure during MNU-induced prostate carcinogenesis in rats. We objectively documented changes in nuclear architecture that correlate with traditional histopathologic criteria and parallel changes in gene expression (AR and p-AKT) as well as phenomena such as proliferation, apoptosis, and vascularity [4]. The observations from the MNU model are similar to those reported in several human studies [30,31,58]. Indeed, greater nuclear area is associated with a poor prognosis [59–61]. In addition to using histone H1 staining as a tool to assess nuclear size and shape, we also quantified changes in chromatin structure within the nucleus (chromatin density). Histone H1 functions as a transcriptional repressor [62] and gene activation requires the removal or rearrangement of H1 histone in order to unpack the compact chromatin fiber in preparation for transcription [62,63]. The changes in chromatin density that we observed and its relationship to genetic damage, chromosomal replication, DNA repair, and gene expression is poorly understood and undoubtedly very complex [64]. However, as a biomarker, chromatin density may be useful in the assessment of interventions designed to slow cancer progression.

In conclusion, we observed predictable changes in nuclear AR expression, p-AKT expression, and nuclear morphometry during the progression of MNU-induced prostate carcinogenesis. These changes occur in parallel with advancing histopathologic grade and stage, alterations in angiogenesis, and the ratio of proliferation/apoptosis previously described in the MNU model [4]. Furthermore, these alterations reflect observations derived from human specimens, providing additional validation of the MNU model as a valuable tool for the preclinical assessment of preventive or therapeutic agents. This system also provides an experimental platform for future studies evaluating the diverse mechanisms whereby androgen insensitivity may develop and assessing how environmental variables (diet, drugs, and hormones) may influence the specific types of genetic changes contributing to androgen insensitivity and p-AKT activation [65]. Changes in nuclear morphometry and

chromatin density provide additional biomarkers reflecting global changes in gene expression and cell biology associated with the prostate cancer cascade. Thus, the MNU model can be employed to better understand the underlying molecular events influencing nuclear architecture, and perhaps provide insight into novel targets for prevention and therapy. Finally, digital image analysis provides an opportunity for investigators to increase objectivity and obtain reproducible and quantifiable biomarker data from histopathologic specimens thereby enhancing the ability to integrate data from various laboratories and assess relevance to human specimens.

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GENISTEIN INHIBITS VEGF-MEDIATED AUTOCRINE AND PARACRINE ANGIOGENESIS NETWORK BETWEEN PROSTATE CANCER CELLS AND VASCULAR ENDOTHELIAL CELLS

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Running title: Genistein Inhibition of VEGF Network

Key words: Genistein, VEGF, HIF-1α, prostate cancer and HUVECs.

Abbreviations:

GAPDH: glyceraladhyde-3-phosphate dehydrogenase
cDNA: complementary DNA
HIF-1α: Hypoxia-inducible factor-1α
VEGF: vascular endothelial growth factor
KDR: kinase insert domain-containing kinase
FLT-1: fms-like tyrosine kinase-1
HUVEC: Human umbilical vein endothelial cells

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ABSTRACT

We have reported that dietary soy products inhibit murine prostate tumor progression in association with a reduction in tumor microvessel density. VEGF stimulates angiogenesis by interacting with fms-like tyrosine kinase-1 (FLT-1) and kinase insert domain-containing kinase (KDR) receptors on endothelial cells. Moreover, tumors are known to be hypoxic and hypoxia can further stimulate tumor angiogenesis through the regulation of VEGF and the transcriptional factor, hypoxia-inducible factor-1α (HIF-1α), as well as VEGF receptors in tumor cells and endothelial cells. The goal of the present study is to investigate the hypothesis that genistein inhibits the autocrine and paracrine activity of VEGF and VEGF receptors in human prostate cancer and vascular endothelial cells. By employing MTS and *in vitro* tube formation assay, we determined that genistein inhibits growth and tube formation of human umbilical vein endothelial cells (HUVECs) stimulated by exogenous VEGF or by hypoxia (5% CO_2, 1% O_2, 94% N_2) exposed PC-3 cell media. Genistein causes a dose-dependent inhibition of VEGF expression in both human prostate cancer PC-3 cells and HUVECs with or without exposure to hypoxia based on RT-PCR and western blotting assays. Furthermore, genistein reduces the expression of HIF-1α in response to hypoxia in PC-3 cells and the expression of Flt-1, but not KDR on the HUVECs. Overall, these observations support the hypothesis that the soy isoflavone genistein may inhibit prostate tumor angiogenesis through VEGF-mediated autocrine and paracrine networks.

INTRODUCTION

Angiogenesis is a multi-step process that is considered essential for tumor development and progression (1). Accordingly, prostate tumors with metastasis show increased microvessel density compared with localized prostate cancer. The microvessel density is increased in prostate intraepithelial neoplasm compared with benign epithelia (2). Therefore, anti-angiogenic therapy has appeared to be a novel and effective strategy for the treatment of prostate cancer. This concept is further supported by the fact that human prostate tumor cells have a remarkably low rate of cell proliferation, thus the inhibition of angiogenesis within prostate tumor may cause hypoxia-induced apoptosis without a requirement of cell proliferation (3).

Epidemiological studies have suggested an inverse relationship between the consumption of soy products and prostate cancer risk (4, 5). Genistein is one of the most biologically active isoflavones in soy products (6). Rodent studies demonstrate that prostate tumor growth is reduced in mice fed diets containing isoflavone rich protein and soy phytochemical concentrates (7, 8). A significant reduction of tumor microvessel density is observed in tumors from mice fed with soy products, suggesting that soy components may inhibit prostate tumor angiogenesis (7). However, the underlying molecular mechanisms by which soy components may inhibit prostate tumor angiogenesis remain to be characterized.

Tumor angiogenesis is regulated by a variety of hormones, growth factors, and cytokines within the tumor microenvironment (9). VEGF has been implicated as one of the most important pro-angiogenic growth factors associated with prostate cancer (10). VEGF, also known as a vascular permeability factor, is essential for normal vasculature development and tumor angiogenesis through the stimulation of endothelial cell proliferation, differentiation, and primitive vessel formation (11). Thus far, six different human VEGF isoforms have been identified due to

alternative RNA slicing, including VEGF121, VEGF145, VEGF165, VEGF183, VEGF189, and VEGF 206 (12). Among VEGF isoforms, VEGF121, VEGF165 and VEGF189 are the most commonly expressed in VEGF-producing cells (13). VEGF121 is a secreted form, whereas VEGF165 and VEGF189 are on the cell surface or interacted with ECM with their basic heparin binding capacities (14). VEGF is known to elicit its biological roles through the interaction with two classes of VEGF receptors, FLT-1 and KDR, which are protein tyrosine kinases (15). Besides the VEGF, HIF-1 is another important factor to regulate tumor angiogenesis. HIF-1α protein is constitutively synthesized and rapidly degraded under normoxic conditions. However, the cellular content of HIF-1α is dramatically increased by hypoxia probably through a combination of increased synthesis and decreased degradation (16-18). Most growing tumors have been suggested to experience hypoxic condition within the tumor microenvironment due to the sluggish blood flow caused by the irregular and poorly formed vasculature (19). The hypoxic microenvironment within tumors enhances levels of HIF-1α, which then up-regulates VEGF expression (20). In addition to the paracrine regulation of angiogenesis by tumor cells, the adjacent endothelial cells may show increased expression of VEGF in response to hypoxia and thus stimulate angiogenesis through an autocrine fashion (21)

In the present *in vitro* study, we test the hypothesis that genistein may play an important role in the inhibition of prostate tumor angiogenesis through regulation of VEGF-mediated paracrine and autocrine stimulated proliferation of endothelial cells.

MATERIALS AND METHODS

Reagents. Synthetic genistein was obtained from Sigma (St. Louis, MO), dissolved in DMSO, and stored as a 20mM stock solution at -20°C. Final working concentrations of DMSO *in vitro* are less than 1%. Human VEGF was obtained from R&D Systems (Minneapolis, MN).

Cell culture. Human prostate cancer PC-3 cells, which are androgen-insensitive, were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI 1640 medium (Life Techologies, Gaithersburg, MD), supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, CA) and maintained as monolayer cultures using a endothelial cell growth medium (EGM-2; Clonetics) containing hydrocortisone, human epidermal growth factor (EGF), VEGF, insulin-like growth factor-1, fibroblast growth factor (FGF), heparin, ascorbic acid, gentamicin and 1% fetal bovine serum (FBS). Cell cultures are maintained at 37°C in a humidified atmosphere of 5% CO_2. For hypoxic treatment, cells are incubated in a sterile chamber flushed with 1% O_2, 5% CO_2, and 94% N_2.

RT-PCR analysis. RNA is extracted using the Absolutely RNA isolation kit (Stratagene, La Jolla, CA). RT-PCR is performed using 1μg of total cellular RNA and the Ribo Clone cDNA synthesis kit (Promega Corp., Madison, WI). The following primers are used for: (a) human VEGF primers: sense (5'-CGA AGT GGT GAA GTT CAT GGA TG-3') and antisense (5'-TTC TGT ATC AGT CTT TCC TGG TGAG-3'); (b) human FLT-1 primers: sense (5'-TGA AGG TCG GAG TCA ACG GAT TTG GT-3') and antisense (5'-CAT GTG GGC CAT GAG GTC CAC CAC-3'). (c) human KDR primers (5'-TGA AGG TCG GAG TCA ACG GAT TTG GT-3') and antisense (5'-CAT GTG GGC CAT GAG GTC CAC CAC-3'). (d) human GAPDH primers obtained from Clontech (Palo Alto, CA) and sequences were as follows: sense (5'-TGA AGG TCG GAG TCA ACG GAT TTG GT-3') and antisense (5'-CAT GTG GGC CAT GAG GTC CAC CAC-3'). The conditions

employed for RT-PCR are as follows: 95°C for 45 sec, 60°C for 2 min, and 72°C for 2 min, 25~28 cycles, 72°C for 7 min, 1 cycle, final extension using DNA thermal cycler (MJ Research INC, Alameda, CA). The integrity of the RNA employed for reverse transcription is assessed using GAPDH synthesis as a positive control reaction. The amplified RT-PCR products are analyzed by electrophoresis on a 1% agarose gel, visualized by ethidium bromide staining and photographed under UV illumination.

Western Blot Analysis. The nuclear extract is prepared as previously described. After electrophoresis, proteins are electro-transferred to PFVD membranes (Bio Rad, Hercules, CA). Complete transfer is confirmed using the prestained protein standards. After blocking in 5% nonfat milk in TBS + 0.05% Tween 20 for 1 hour at room temperature, the membrane is incubated with anti-human mouse monoclonal HIF-1α67 (Novus Biologicals Inc., Littleton, CO) at 1 μg/ml. The membrane is subsequently incubated with the HRP-linked secondary antibodies for 30 min, followed by application of luminol reagents and final detection by ECL system (Santa Cruz Biotech., Santa Cruz, CA).

Immunohistochemical Staining. Cells are treated with 1% O_2 following pretreatment with either vehicle or genistein for 72 hours. Cells are then harvested, mixed with thrombin and plasma to form a matrix that is then fixed in 10% formalin and paraffin embedded to form cell blocks. Immunohistochemical staining was then conducted by employing ABC kits (Santa Cruz Biotech., Santa Cruz, CA). Briefly, sections are cut and mounted on slides. Sides are then washed and blocked with 10% goat blocking serum, and incubated overnight at 4°C with mouse monoclonal anti-human HIF-1α antibody (Novus Biologicals, Inc, Littleton, CO). After PBS washing, slides are incubated with biotinylated anti-mouse secondary antibody for 30 min and are subsequently treated with the biotin-avidin-horse-radish peroxidase complex, followed by the color detection with DAB (diaminobenzidine), and counterstaining with hematoxylin.

***In vitro* Cell Proliferation Assay.** A total of 4 X 10^3 cells are plated per well of 96-well microplates. After 24 hours, cells are treated with vehicle or genistein in the presence or absence of 10ng/ml VEGF for 72 hours. Cell proliferation is determined by the CellTiter 96 Aq_{ueous} One Solution Cell Proliferation Assay kit (Promega Corp., Madison, WI). In brief, the MTS assay is an indirect measurement of viable cells based upon the ability of dehydrogenase enzymes in metabolically active cells to cleave the tetrazolium salt MTS, [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], and yield a highly colored, water soluble formazan product. The addition of the electron coupling agent phenazine ethosulfate (PES) potentiates the colorimetric reaction. All assays are completed in triplicate, experiments replicated, and results confirmed by direct cell counting using a hemocytometer.

***In vitro* Tube Formation Assay.** *An in vitro* tube formation assay is performed employing the *In Vitro* Angiogenesis Assay Kit (Chemicon International, Temecula, CA). Each well of 96-well tissue culture plate receives 50 µl of extracellular matrix solution. Following a brief incubation at 37°C, 5,000 of HUVEC are added on top of the solidified matrix solution. The cells are then treated with vehicle or genistein in the presence or absence of 10 ng/ml VEGF. The tube formation is evaluated by an inverted light microscope at 10X magnification after overnight incubation at 37°C. Photographs are taken of each well and quantitated.

Statistical Analysis. Results of RT-PCR and Western blotting are photographed and evaluated by densitometry (Alpha Innotech Corporation, San Leandro, CA). Data from flow cytometry and densitometric analysis of expression of mRNA and protein are initially evaluated by ANOVA followed by Fisher's protected least significant difference (PLSD) test to evaluate pairwise comparisons among treatment groups using the Statview 4.5 (Abacus Concepts,

Berkeley, CA). The number of replicates for each study is detailed in the figure legends, tables, or result sections. A probability level of $p < 0.05$ is termed significant.

RESULTS

Genistein Inhibits the Proliferation of HUVECs with or without VEGF-Stimulation. To investigate whether genistein may directly reduce the growth of endothelial cells, the HUVEC growth is analyzed by MTS assay after incubation with genistein at the presence or absence of VEGF. Without the presence of VEGF, genistein dose dependently inhibits the proliferation of HUVECs with an IC_{50} below 20 μM (Fig 1). As expected, VEGF at 10ng/ml significantly increases the growth of HUVECs to 128% ($p<0.01$) of the control (Fig.1). Treatment with genistein induces a dose-dependent growth inhibition of HUVECs even under VEGF stimulation. For example, genistein at 10 μM, 20 μM, 50 μM, and 100 μM, inhibits the VEGF-stimulated growth of HUVEC by 16% ($p<0.01$), 54% ($p<0.001$), 70% ($p<0.0001$) and 72% ($p<0.0001$), respectively (Fig.1).

The *in vitro* HUVEC tube formation in a 96-well matrix bottomed plates provides a good model to examine the effects of angiogenic and anti-angiogenic factors. Our results show that exposure to VEGF (10ng/ml) increases the tube formation of HUVEC compared to the control. Genistein at the concentration of 50 μM inhibits the HUVEC tube formation with or without VEGF stimulation (Fig 2).

Genistein Modulates the mRNA Expression of VEGF and VEGF Receptors (FLT-1 and KDR) of HUVECs under both Normoxic and Hypoxic Conditions. To investigate whether genistein inhibits VEGF mediated autocrine effects on endothelial cells, VEGF, FLT-1, and KDR mRNA expression is examined in HUVECs treated with genistein. Fig 3A is a representative RT-PCR result showing that genistein significantly decreases VEGF mRNA expression but causes no significant changes of FLT-1 and KDR mRNA expression under normoxic condition. Densitometric evaluation of genistein-treated cells compared to vehicle-treated cells shows that genistein at 10 μM and 50 μM inhibits VEGF by 28% ($p<0.01$), and 41% ($p<0.001$), respectively.

VEGF, FLT-1, and KDR mRNA expression in HUVECs are further examined with the treatment of genistein under hypoxic condition (1% O_2). Fig 3B is a representative RT-PCR blot showing that hypoxia up-regulates VEGF and FLT-1 mRNA expression and that genistein at 10 μM and 50 μM inhibits these responses in a dose-dependent manner. Densitometric evaluation shows that an exposure for 24 h to 1% O_2 induces 2.4-fold ($p<0.001$), 2.3-fold ($p<0.001$) increase of VEGF and FLT-1 mRNA in HUVECs, respectively and that genistein at 10 μM and 50 μM causes an inhibition of hypoxia-induced VEGF mRNA expression by 21% (0.01) and 28% ($p<0.01$), respectively. Genistein causes a dose-dependent inhibition of FLT-1 mRNA at 10 μM by 61% ($p<0.001$) and 50 μM by 70% ($p<0.001$). No significant changes of KDR mRNA expression are observed in HUVECs under hypoxic condition with or without the treatment of genistein.

Genistein Inhibits Proliferation of HUVECs with Paracrine Stimulation of Prostate Cancer Cells. In order to examine whether prostate cancer cells causes a paracrine stimulation to proliferation of endothelial cells, the proliferation of HUVECs is analyzed following exposure to conditioned media from PC-3 cells after hypoxic incubation for 24h. As shown in Fig. 4, the conditioned media from hypoxic PC-3 cells stimulates the proliferation of HUVEC by 28% ($P<0.01$) compared with normoxic PC-3 cell media. Treatment with genistein elicits a dose dependent growth inhibition of HUVECs stimulated by under conditioned media from hypoxic PC-3 cells. Genistein at 10, 20, 50 and 100 μM significantly inhibit the conditional media stimulated growth of HUVECs by 16% ($P<0.01$), 54% ($P<0.001$), 70% ($P<0.001$) and 72% ($P<0.0001$), respectively.

Genistein Modulates VEGF and HIF-Iα Expression in PC-3 Cells under Normoxic and Hypoxic Condition. To investigate whether genistein modulates the VEGF-mediated paracrine stimulation of PC-3 cells, the expression of VEGF and HIF-1α in PC-3 cells is further

examined in HUVECs with the addition of genistein under normoxic and hypoxic condition. As shown in Fig. 5A, genistein dose-dependently down-regulate VEGF mRNA expression in PC-3 cells. Densitometric evaluation (n=3) of Western blot analysis demonstrates that compared to PC-3 cells treated with vehicle (density =1), genistein-treated cells have the density of VEGF165 of 0.74 at 10 μM ($p<0.001$), 0.65 at 20 μM ($p<0.001$) and 0.54 at 50 μM ($p<0.001$). The effect of genistein inhibition of VEGF-mediated paracrine function of PC-3 cells is further examined under hypoxic conditions. VEGF mRNA expression is examined in PC-3 cells treated with genistein under hypoxia condition (1% O_2). Figure 5B is a representative RT-PCR gel showing that hypoxia (1% O_2) exposure significantly up-regulates VEGF mRNA expression and that genistein inhibits this response in a dose-dependent manner. Densitometric evaluation shows that compared to control (density = 1), hypoxia (1% O_2) exposure for 24 h induces a 4.6-fold increase ($p<0.0001$) in VEGF165 mRNA in PC-3 cells and that genistein treatment at 10 μM and 50 μM causes inhibition of hypoxia-induced VEGF165 mRNA expression by 27% ($p<0.01$) and 51% ($p<0.001$). This finding is confirmed by a Western blot analysis of hypoxia-exposed PC-3 cells with genistein treatment (Fig 5C). Densitometric analysis demonstrates that compared to control (density = 1), hypoxia treatment significantly increases VEGF protein expression (density = 2.4 ± 0.5, $P<0.001$) and genistein treatment causes a dose-dependent inhibition of this induction. With the treatment of genistein at 10 or 50 μM, the protein levels of VEGF in PC-3 cells are reduced to 71% ($p<0.01$) and 46% ($P<0.001$), respectively, compared with the hypoxic condition alone ($p<0.001$).

In order to investigate the molecular mechanisms whereby genistein inhibits hypoxia-induced VEGF expression, the expression of HIF-1α mRNA and protein is examined in PC-3 cells with the treatment of genistein under normoxic and hypoxic conditions. Genistein does not reduce the mRNA expression of HIF-1α in PC-3 cells under normoxic condition (Fig. 5A). Hypoxia exposure causes no significant change of HIF-1α mRNA expression and treatment with

genistein at the concentrations of 10 or 50 μM has no effect for the HIF-1α mRNA expression in PC-3 cells (Fig. 5B). However, a Western blot analysis of nuclear extract shows an increase in HIF-1α expression in hypoxia-exposed PC-3 cells (Fig. 5C). Densitometric analysis demonstrates that compared to control (density = 0), hypoxia treatment significantly induces HIF-1α protein expression and genistein treatment causes a dose-dependent down-regulation of this induction at 10 μM by 26% ($p<0.01$) and 50 μM by 54% ($p<0.001$). The increased protein expression of HIF-1α under hypoxia is further supported by the change of hexokinase II protein, a HIF-1α response gene, in PC-3 cells. Hypoxia exposure increases the expression of HKII by 32% ($P<0.01$) compared with the control. Genistein shows dose dependent inhibition of HKII expression in PC3 cells by 17% ($P<0.05$) and 43% ($P<0.001$) at the concentrations of 10 and 50 μM, respectively, compared with the hypoxic condition alone (Fig. 5C). The immunohistochemical staining also shows a significant increase in the nuclear staining of HIF-1α in PC-3 exposed to hypoxia (87%) compared with control (0) ($P<0.001$) (Fig. 6). Genistein causes a dose dependent decrease in nuclear staining of HIF-1α in PC-3 cells under exposure to hypoxia. Only 56% and 25% of cells are stained with treatment of genistein at 10 and 50 μM, respectively (both $P<0.01$ compared with the hypoxic condition).

DISCUSSION

Despite the apparent association of soy consumption and decreased prostate cancer risk, the critical molecular mechanisms whereas soy products inhibit prostate tumor growth are not fully elucidated. Most efforts during the past decades tried to examine the effects of soy products on prostate tumor incidence and growth in animal models (22). The effects of soy products on prostate tumor angiogenesis are poorly understood. Our current *in vitro* study investigates the molecular mechanism of genistein inhibition of prostate tumor angiogenesis. Our results indicate that genistein exerts its anti-angiogenic effect via the inhibition of VEGF-mediated paracrine and autocrine angiogenesis networks between prostate cancer cells and endothelial cells.

Our first observation is that the genistein is able to inhibit proliferation and tube formation of endothelial cells with or without VEGF stimulation, implicating a potential role for inhibition of prostate tumor angiogenesis. The inhibitory effects of genistein on the proliferation of endothelial cells have been reported by prior researchers (23). Genistein has been shown to be the most potent inhibitor of endothelial cell proliferation and *in vitro* angiogenesis, among the active components fractionated from urine of human consuming a plant-based diet (24).Our previous rodent studies have demonstrated that soy protein and soy phytochemical concentrate significantly reduce microvessel density in transplanted prostate tumors (7). This anti-angiogenic effect of genistein has also been shown in transplanted Lewis lung cancer and B16 melanoma in mice (25). Our studies suggest that genistein is a very promising reagent for prostate cancer chemoprevention and chemotherapy due both to its anti-angiogenic and anti-cancer effects.

Our study further elucidates the mechanism whereby genistein may inhibit angiogenesis. Genistein inhibits the angiogenesis through the reduction of paracrine VEGF produced by prostate cancer cells and the autocrine release by endothelial cells themselves. It therefore

inhibits the VEGF-mediated paracrine or autocrine angiogenesis network between prostate cancer cells and vascular endothelial cells. VEGF is a potent pro-angiogenic factors with several different isoforms (26). The finding that genistein inhibits VEGF expression is consistent with a series of published studies. Genistein (100 μg/ml) significantly inhibits the epidermal growth factor and hypoxia induced expression of VEGF in human SMKT-R-1 and SMKT-R-3 renal cell carcinoma cell lines (27). Genistein has also been shown to inhibit the hypoxia-induced VEGF mRNA expression in kidney 293 cells, U87 glioma cells, and HT1080 human fibrosarcoma cells (28).

VEGF receptors play important role in mediating VEGF function. Our finding in the present study is that hypoxia exposure alters mRNA expression of the specific VEGF receptors, FLT-1, but not of KDR, on cultured endothelial cells. The enhanced expression of FLT-1 mRNA in HUVEC with hypoxia exposure is decreased with the treatment of genistein. In addition to regulation of VEGF receptors, genistein may also modulate VEGF functions through a signal transduction pathway. VEGF treatment has been shown to induce the tyrosyl phosphorylation of KDR/Flk-1, and activation of p44/p42 MAPK and AKT pathways (29-32), implicating an important role of signal transduction in mediating VEGF functions. Data from our laboratory and other groups have shown that genistein can cause inhibition of the tyrosyl phosphorylation and the phosphorylation of AKT and p44/p42 MAPK proteins in prostate cancer and other cells (33). Intracellular signaling pathways regulating VEGF may also be the possible targets for the anti-angiogenic effect of genistein.

Due to the prevalence of hypoxic condition in prostate tumors, the transcription factor HIF-1α plays an important role in prostate tumor angiogenesis indirectly through upregulation of VEGF expression. HIF-1α protein is constitutively synthesized and rapidly degraded by the ubiquitin-proteasome pathway under normoxic conditions. However, the cellular content of HIF-

1α is dramatically increased by hypoxia, probably through a combination of increased synthesis and decreased degradation (16-18). We demonstrate that genistein causes a dose-dependent decrease of hypoxia-induced HIF-1α mRNA and protein expression. This finding provides an insight into the molecular mechanism indicating that genistein may decrease hypoxia induced-VEGF expression through HIF-1α in prostate tumor cells and thus inhibit prostate tumor angiogenesis. However, genistein reduces VEGF mRNA expression without effect on the HIF-1α under normoxic condition, suggesting that genistein also inhibits VEGF expression in a HIF-1α independent manner. Consistent to this finding, genistein has been reported to inhibit angiogenesis in pancreatic cancer through inhibition of HIF-α (34). Genistein inhibits hypoxia induced HIF-1α synthesis and HIF-1α DNA-binding activity in Hep3B cells (35). Previous studies have suggested that PI3K pathway is essential for the stabilization of HIF-1α protein (36). The inhibition of PI3K pathway may be one of the mechanisms attributing to the genistein effect on HIF-1α.

Overall, we hypothesize that prostate tumors exhibit enhanced angiogenesis as indicated by the increased proliferation of endothelial cells at the hypoxic core due to the elevated VEGF production from both tumor cells and endothelial cells themselves (in response to hypoxia). The soy isoflavone, genistein, inhibits prostate tumor angiogenesis through the regulation of VEGF and VEGF receptor expression via both autocrine and paracrine pathways in endothelial cells and prostate cancer cells.

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FIGURE LEGENDS

Figure 1. Genistein inhibits the proliferation of HUVECs with or without VEGF-stimulation. HUVECs are treated with increasing concentrations of genistein in the presence or absence of VEGF (10ng/ml) for 72 h. The cell viability of HUVECs is examined by MTS assay (n=9). VEGF significantly stimulates the growth of HUVECs and genistein inhibits the proliferation of HUVECs with or without VEGF stimulation.

Figure 2. Genistein reduces *in vitro* HUVEC tube formation with or without VEGF stimulation. HUVECs are seeded on 96-well matrix bottomed plates and treated with (genistein 50 μM) in the presence or absence of VEGF (10 ng/ml). The tube formation of HUVEC is evaluated by *in vitro* angiogenesis assay as described as in "materials and methods" (n=3). VEGF at 10ng/ml significantly increases the tube formation of HUVECs and genistein at 50 μM reduces the tube formation with or without VEGF stimulation.

Figure 3. Genistein modulates mRNA expression of VEGF and VEGF receptors (KDR and FLT-1) of HUVECs under normoxic and hypoxic conditions. HUVECs are treated with designated concentrations of genistein for 72 hours and total RNA is isolated and analyzed by RT-PCR (n=3). (A) Genistein causes a dose-dependent inhibition of VEGF mRNA expression but not FLT-1 and KDR mRNA expression in HUVECs under normoxic condition. (B) Both VEGF and FLT-1 mRNA expression is enhanced by hypoxic exposure. Addition of genistein inhibits this response with a dose dependent manner. No response is observed for the KDR receptor under above treatment.

Figure 4. Genistein decreases the proliferation of HUVECs incubated with conditioned media from PC-3 cells after normoxic and hypoxic exposure for 24 h. HUVECs are seeded on the 96-well plates and treated with conditioned media at the presence or absence of genistein for 48 h. The HUVEC viability is evaluated by the MTS assay (n=9). The conditioned media from PC-3 cells exposed to hypoxia significantly stimulates the growth of HUVEC growth by 28%. Genistein shows dose dependent inhibition of the growth of HUVECs incubated with both conditioned media.

Figure 5. Genistein modulates VEGF and HIF-1α expression in PC-3 cells under normoxic and hypoxic conditions. (A) Genistein reduces the mRNA expression of VEGF but not HIF-1α in PC-3 cells under normoxic conditions. PC-3 cells are treated with genistein with designated concentrations for 24 h. Total RNA is extracted and the mRNA expression is quantified after RT-PCR (n=3). (B) Genistein reduces the mRNA expression of VEGF and HIF-Iα in PC-3 cells under hypoxic conditions. PC-3 cells are exposed to 1% O_2 for 24 h with or without pre-treatment of genistein at 10 μM and 50 μM. Total RNA is isolated and mRNA expression is analyzed after RT-PCR (n=3). Hypoxia exposure induces of VEGF mRNA expression in both PC-3 cells. Genistein inhibits this response at a dose-dependent manner. HIF-Iα mRNA expression shows no response to the above treatment. (C) Genistein inhibits hypoxia-induced VEGF, HIF-1α and hexokinase II protein expression in PC-3 cells. Cells are exposed with 1% of O_2 for 24 h with or without pre-treatment of genistein at 10 μM and 50 μM. Whole cell lysate or nuclear extract (for HIF-1α) is prepared and analyzed for western blot analysis (n=3). Hypoxia exposure causes an induction of VEGF, HIF-1α and hexokinase II protein expression PC-3 cells. Genistein at 10 μM and 50 μM causes a dose-dependent inhibition of the response of these three proteins.

Figure 6. Immunohistochemical staining shows genistein inhibition on hypoxia-induced HIF-1α protein expression in PC-3. Cells are exposed with 1% of O_2 for 24 h with or without pre-treatment of genistein at 10 and 50 μM. The expression of HIF-1α is examined by immunohistochemical staining (n=3). Hypoxia exposure causes an induction of nuclear brown staining of HIF-1α. Treatment with genistein at 10 and 50 μM causes a dose-dependent decrease of HIF-1α staining.

Figure 1

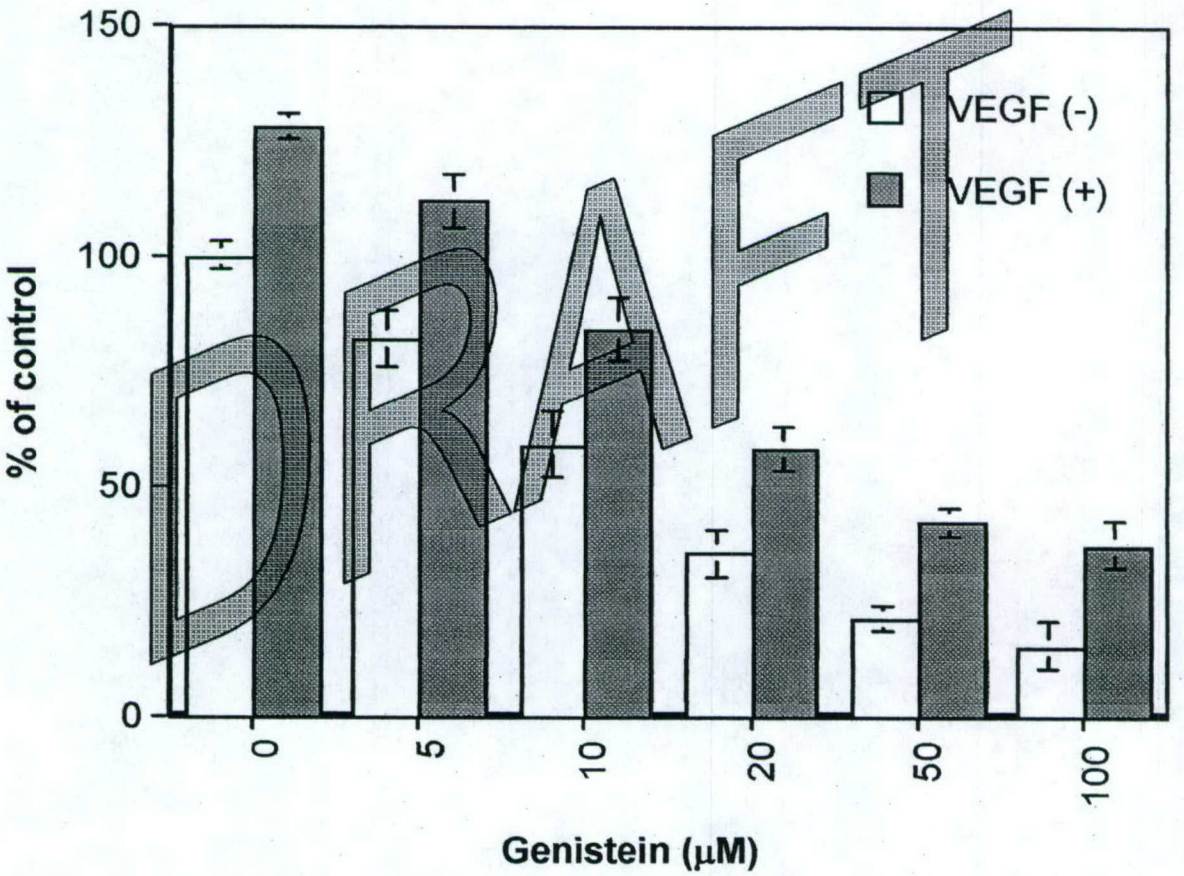


Figure 2

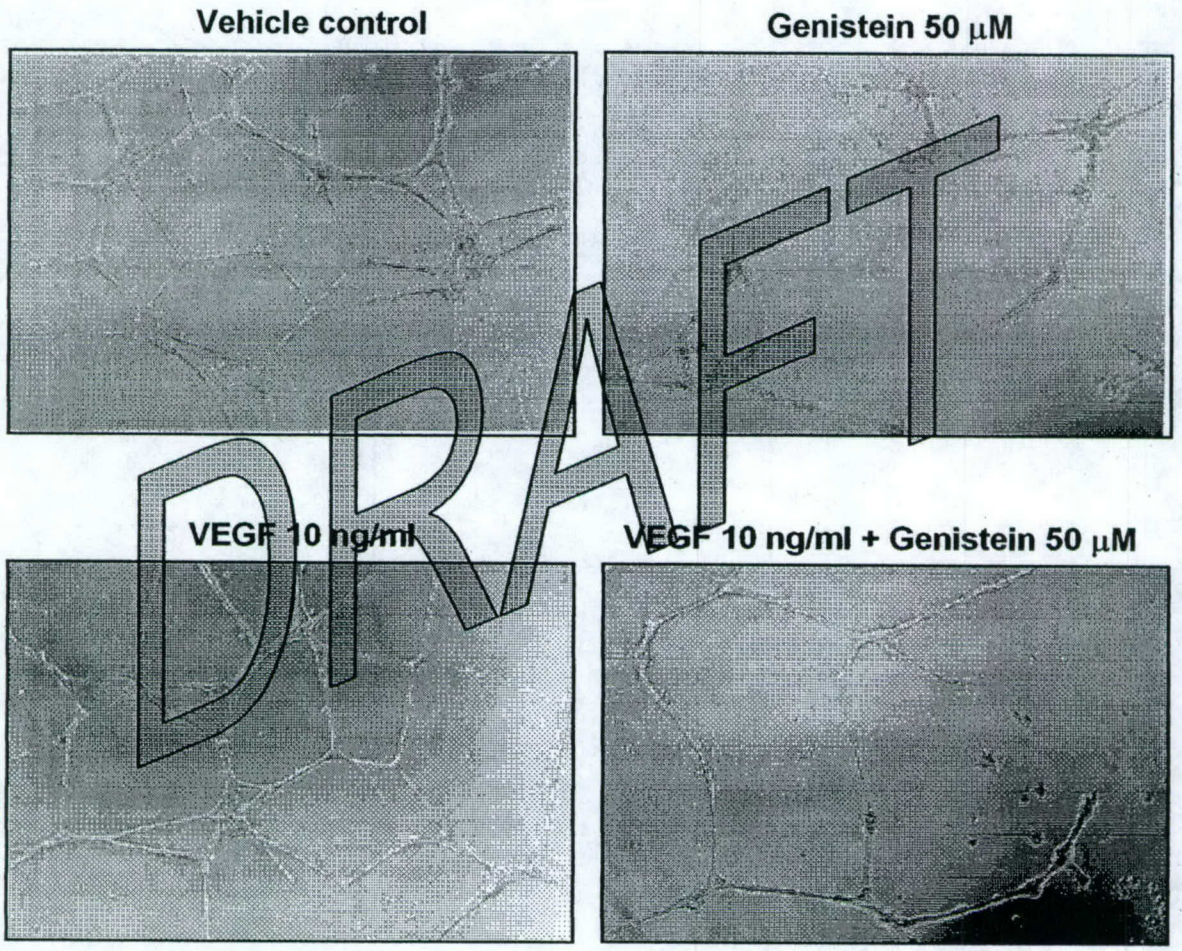
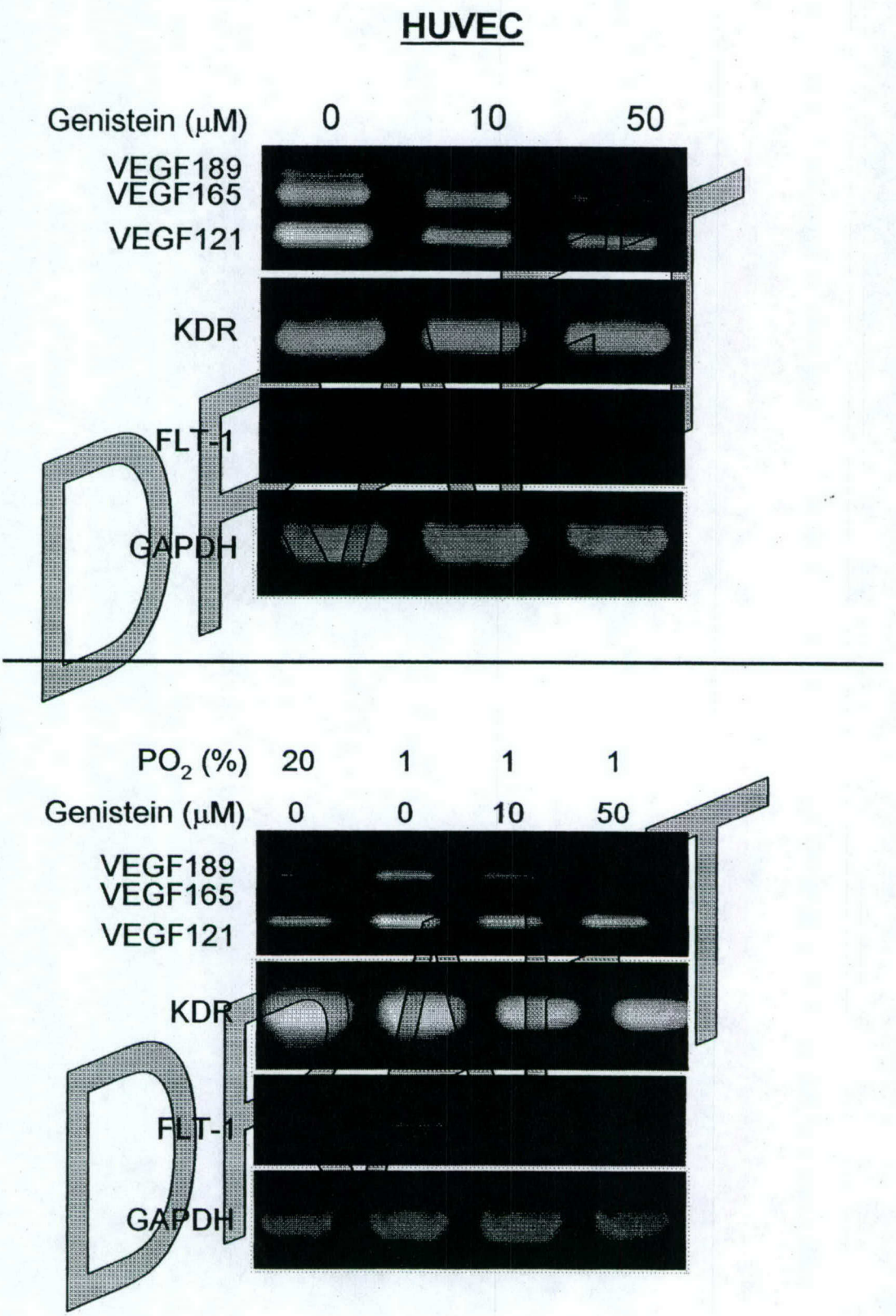


Figure 3


A
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Genistein (μM)
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VEGF189
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KDR
FLT-1
GAPDH
B
PO2 (%)
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1
1
1
Genistein (μM)
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VEGF121
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Figure 4

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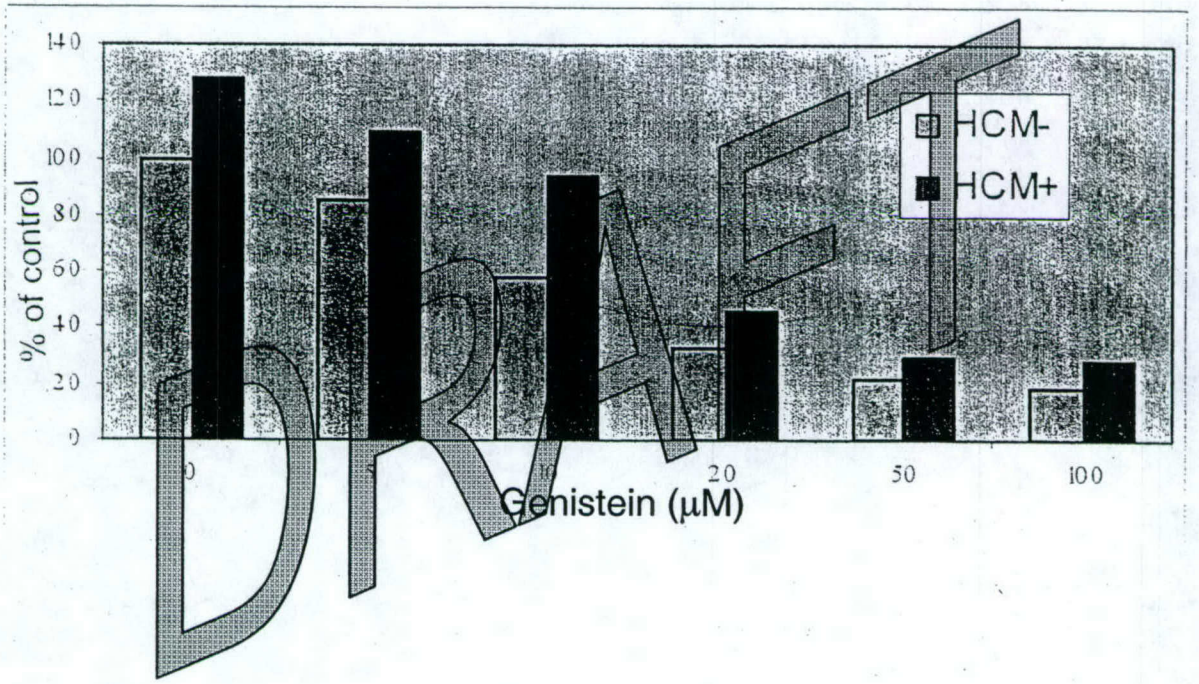

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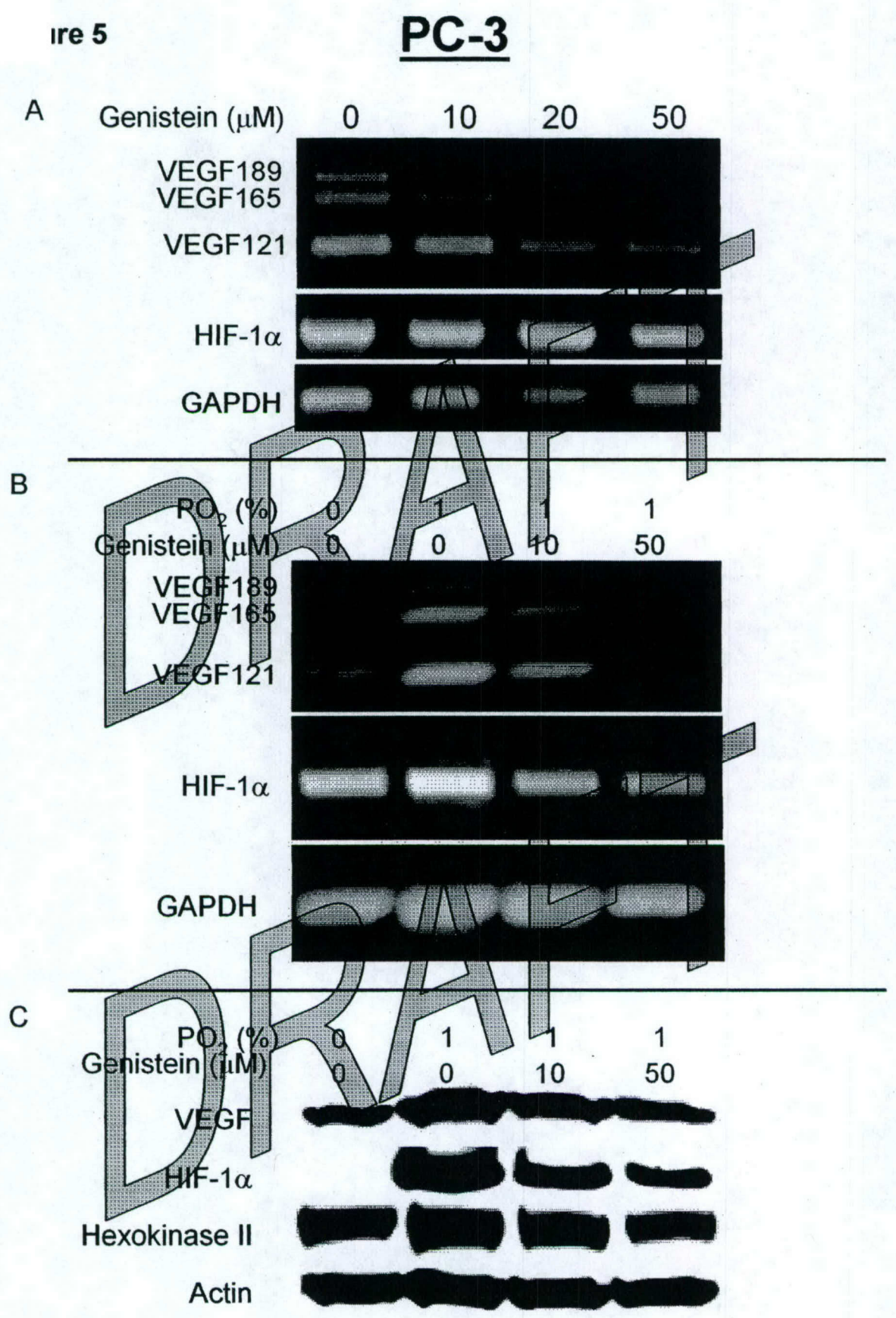
PC-3
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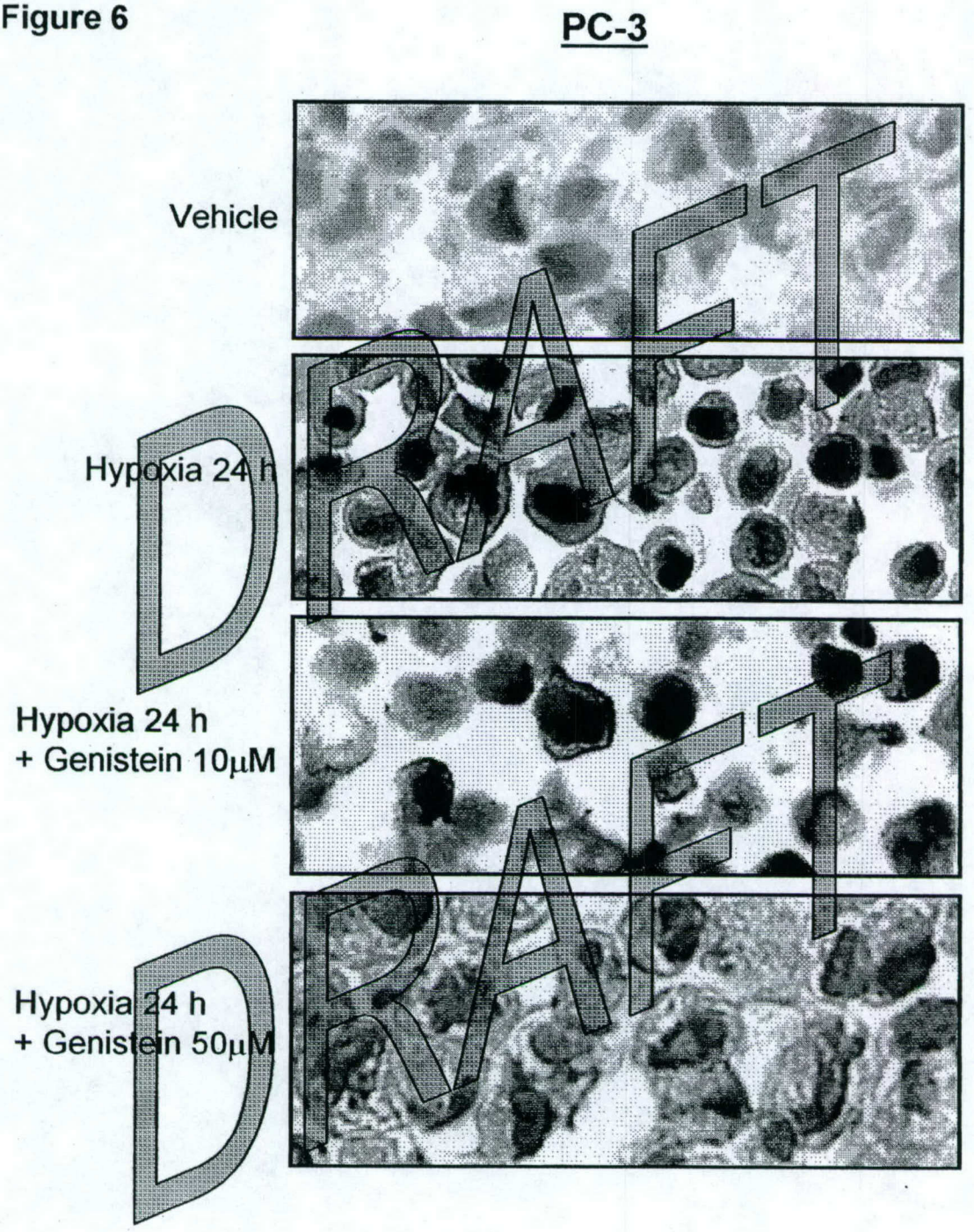
VEGF189
VEGF165
VEGF121
HIF-1α
GAPDH
C
PO2 (%) 0 1 1 1
Genistein (μM) 0 0 10 50
VEGF
HIF-1α
Hexokinase II
Actin

Figure 6


PC-3
Vehicle
Hypoxia 24 h
Hypoxia 24 h
+ Genistein 10μM
Hypoxia 24 h
+ Genistein 50μM
DRAFT
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Dietary Information Sheet

Name_____ Date_____

Address_____ Phone: (H)_____

_____ (W)_____

Height_____ Weight_____ E-mail_____

What is the best way to contact you?_____

Subject #_____
(*Investigator Use Only*)

*We will contact you to set up your individual appointment

How frequently do you consume tomatoes?
___never ____daily ____biweekly ____weekly ____twice/month _____monthly

How many servings/day? (1 serving =1/2 tomato)_____

How frequently do you consume tomato juice (not V8 or vegetable juice)?
___never ____daily ____biweekly ____weekly ____twice/month _____monthly

How many servings/day? (1 serving = 8oz) _____

How frequently do you consume V8 juice?
___never ____daily ____biweekly ____weekly ____twice/month _____monthly

How many servings/day? (1 serving = 5oz) _____

How frequently do you normally consume tomato soup?
___never ____daily ____biweekly ____weekly ____twice/month _____monthly

How many servings/day? (1 serving = 8oz) _____

How frequently do you consume pizza?
___never ____daily ____biweekly ____weekly ____twice/month _____monthly

How many servings (slices)/day? (1serving = 1slice)_____

How frequently do you consume red pasta sauce (spaghetti sauce, tomato paste)?
___never ____daily ____biweekly ____weekly ____twice/month _____monthly

How many servings/day? (1 serving = ½ cup) _____

How frequently do you consume watermelon?
___never ____daily ____biweekly ____weekly ____twice/month _____monthly

How many servings/day? (1 serving = ½ cup) _____

How frequently do you consume pink grapefruit or pink grapefruit juice?
___never ____daily ____biweekly ____weekly ____twice/month _____monthly

How many servings/day? (1serving = ½ cup)_____

How frequently do you consume blood oranges?
___never ____daily ____biweekly ____weekly ____twice/month _____monthly

How many servings/day? (1 serving = ½ cup)_____

How frequently do you consume salsa?
___never ____daily ____biweekly ____weekly ____twice/month _____monthly

How many servings/day? (1 serving = ¼ cup)_____

How frequently do you consume ketchup or barbecue sauce?
___never ____daily ____biweekly ____weekly ____twice/month _____monthly

How many servings/day? (1 serving = 1 tbs.)_____

How frequently do you consume soy milk?
___never ____daily ____biweekly ____weekly ____twice/month _____monthly

How many servings? (1 serving = 8 fl. ounces or 1 cup)_____

How frequently do you consume soy burgers (for example Boca Burgers)
___never ____daily ____biweekly ____weekly ____twice/month _____monthly

How many servings per week? (1 serving = 1 burger) _____

How frequently do you consume soy nuts?
___never ____daily ____biweekly ____weekly ____twice/month _____monthly

How many servings? (1 serving = ½ cup) _____

Do you consume any other soy products regularly? Examples include soy beans, soy powder (mixed with milk), soy bars, and tofu. Please list the soy product, serving size and frequency of consumption.

Soy product	Serving Size	How often?
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Tomato Worksheet

Name:_____ **Week** ___ **Day** _____

The foods below have been assigned points according to portion size and nutrient content. Each day, your goal is to consume combinations of the foods listed below so that you accumulate 25 points. You may use any combination of foods, as long as you adhere to the portion sizes and point system below.
You may eat the same food more than one time each day, just make sure you check the appropriate number of boxes on the worksheet.

Food	Serving Size	# of servings 1 2 3 4 5		Points / serving		Total
Tomato, raw	1 whole		x	4	=	
Tomato, raw, slice	2 slices		x	1	=	
Tomato, raw, chopped	½ cup		x	3	=	
Tomatoes, ckd / stewed	1/2 cup		x	5	=	
Tomato, canned / whole	1/2 cup		x	13	=	
Tomato Sauce	½ cup		x	21	=	
Tomato Paste	1 Tbsp.		x	9	=	
Tomato Juice	1 cup / 8 fl. oz		x	21	=	
Tomato Soup	½ cup (condensed)		x	12	=	
Catsup	2 tsp		x	3	=	
Chili	1 cup		x	5	=	
Pizza Sauce (canned)	½ cup		x	16	=	
Pizza slice	1 slice		x	3	=	
Salsa	2 Tbsp.		x	4	=	
Sun Dried Tomato	1 tomato		x	1	=	
Steak Sauce	1 Tbsp.		x	3	=	
Barbecue Sauce	2 Tbsp.		x	1	=	
Vegetable Juice	½ cup		x	10	=	

Soy package 1 ☐
Soy package 2 ☐

Total for Day = _____

Goal = 25 points

SOY GROUP

Name:_____ Day: _____ Date: _____ Week: ___

Soy Powder: Place an "X" in the box when you have consumed each packet of soy.

Package 1	☐
Package 2	☐

Tomato Foods: If desired, each day you may consume ONE serving of the tomatoes and tomato products from the list below. Please place an "X" in the appropriate box and abstain from any tomato food or serving size not on this list.

One raw tomato	☐	1 slice pizza	☐
Tomato slices (6 or fewer)	☐	Sun-dried tomatoes (5 or fewer)	☐
½ cup raw, chopped tomato	☐	1 tablespoon steak sauce	☐
½ cup cooked tomatoes	☐	1 tablespoon BBQ sauce	☐
2 teaspoons ketchup	☐	2 tablespoons salsa	☐
1 cup chili	☐		

Additional Tomato Foods: If you accidentally consumed any tomato or soy-containing foods which are not listed above, please list the food and the amount consumed in the space below.

_____

_____

_____